AN UNCONVENTIONAL ROLE FOR THE SEPTATE JUNCTIONS AND GLIOTACTIN IN CELL DIVISION

by

KRISTI CHARISH

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Abstract

The focus of this thesis is to investigate the integration of cell division with the septate junction domain in the Drosophila imaginal wing disc epithelium. Columnar epithelia of the imaginal wing disc exhibit complex architecture due to an elaborate series of junctions that are found throughout the membrane. During cell division, these junctions are maintained while new junctions are established; however, their role and influence during mitosis is unclear. This thesis shows that the septate junctions are essential for cytokinesis and Gliotactin at the tricellular junctions is necessary to localize cell division to the septate junction domain, and illustrates a unique role for Gliotactin and the septate junctions outside their classic role of maintaining a permeability barrier.

The septate junctions are basolaterally localized transmembrane junctions required in epithelial cells to form a permeability barrier. Although the septate junctions are formed by a large protein complex, this thesis only investigates the three core SJ proteins, NeurexinIV (NrxIV), Coracle (Cor), and Neuroglian (Nrg). Gliotactin (Gli), a Drosophila Neuroligin homologue, is a septate junction associated protein concentrated at the tricellular junction (TCJ), which is necessary to maintain the septate junction permeability barrier. Loss of any of the septate junction proteins, or Gliotactin, leads to structural disruption of the septate junctions and loss of the permeability barrier in a wide range of epithelial derived tissues.

Chapter two examines the process of cell division in epithelial cells of the wing imaginal disc with respect to the septate junctions and tricellular junction. Chapter three looks at the role of Gliotactin in maintaining the plane of cell division within the septate junction domain, and chapter four shows that the septate junctions are necessary for ingression furrow stability and the association of the contractile ring with the membrane during late cytokinesis. This work
demonstrates a novel role for the septate and tricellular junctions during mitosis in Drosophila, which has implications for the role of tight junctions in vertebrate cells.
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List of Abbreviations

Genes
Abbreviations
Septate Junctions
Gliotaclin  
Neurexin IV  
Neuroglian  
Coracle  
Fascilin III  
Discs large  
Lethal giant larvae  
Scribble  

Cell division markers
non-muscle myosin II heavy chain  
Polo like kinase  
Jupiter/ MAP  
Phosphorylated Histone 3  
γ-tubulin  
α-tubulin  
acetylated-tubulin  

Terms
Microtubule associated proteins  
Septate junction  
pleated septate junctions  
smooth septate junction  
Tricellular junction  
Paranodal septate junction  
Tight junction  
Tricellular tight junction  
mosaic analysis with a repressible marker  
Flip Recombinase Target  
Flip Recombinase  

MAPs  
SJ  
pSJ  
sSJ  
TCJ  
PSJ  
TJ  
tTJ  
MARCM  
FRT  
FLP/Flipase
There are a lot of people to thank for their support over the past 6 years but my preference is to keep this short and sweet. First and foremost I have to thank my supervisor Vanessa Auld for her guidance and unwavering support of my research pursuits over the past six years, but mostly for putting up with me and my relentless questions. I also need to thank Murry Gilbert for her help and guidance; I probably would have quit about three years ago. Thank you to all Auld lab members -past and present- for your support and friendship, and as well to my committee for helping get my thesis defence ready.

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I. Introduction

Septate and tricellular junctions in Drosophila polarized epithelial are necessary for maintaining and establishing permeability barriers. In columnar epithelia of the imaginal wing disc, the plane of cell division is localized to the basolateral septate junction domain; however it is unknown whether the septate junctions are necessary for this localization or play a role during cell division.

Introduction to permeability barrier junctions

Specialized junctions form permeability barriers between cells and are responsible for maintaining distinct solute environments between membrane bound compartments. They also prevent the entrance of pathogens. When the permeability barrier is disrupted, solutes can pass across the membrane disrupting the equilibrium. An example of an essential permeability barrier is the blood-nerve barrier, necessary to prevent external solutes from entering the nervous system. For example, a disruption of the permeability barrier during Drosophila embryogenesis leads to paralysis and death (Auld et al., 1995; Baumgartner et al., 1996) and in enclosed tissues, such as the salivary gland, disruption of the permeability barrier leads to an inability for the lumen to keep out external solutes (Auld et al., 1995; Genova and Fehon, 2003; Paul et al., 2003) (Figure 1.2A).

Two types of permeability barriers are the Drosophila septate junctions (SJs) (Hortsch and Margolis, 2003) and the vertebrate tight junctions (TJs) (Tsukita et al., 2001) and both result in tight seals between adjacent cells. The Drosophila SJs form strands that wrap around the membrane and form an electron dense barrier that stretches between neighbouring cells (Hortsch
and Margolis, 2003). The TJs maintain close contact points with their neighbours, blocking diffusion across the membrane and facilitating cell-cell adhesion (Tsukita et al., 2001).

**Septate junctions**

This thesis concentrates on the SJs, which form the primary permeability barrier in Drosophila. They assemble late in Drosophila embryogenesis and are found in a wide range of epithelial tissues (Tepass and Hartenstein, 1994; Tepass et al., 2001). In columnar epithelia, the SJs occupy the apical-lateral region of the membrane directly below the zonula adherens (ZA) (Figure 1.1A, red). The adherens junctions in the zonula adherens form an adhesive belt that encircles the cell directly below the apical surface (Figure 1.1A, yellow) and is associated with an electron dense cytoplasmic plaque of actin (Knust and Bossinger, 2002). Uniformly spaced between cell membranes are septae, the electron dense material that compose the septate junctions (Figure 1.1C,D). The organization and uniform alignment of the septae between adjacent cells give the SJ the appearance of a ladder with rungs (Tepass and Hartenstein, 1994).

There are two types of SJs found in Drosophila; pleated and non-pleated septate junctions (Tepass and Hartenstein, 1994). Non-pleated, or smooth septate junctions (sSJs) are found in endodermally derived tissue such as the midgut, whereas pleated septate junction (pSJs) are found in ectodermally derived tissue such as the foregut, trachea, glia and imaginal discs (Dallai, 1976; Noirot-Timothee and Noirot, 1980; Tepass and Hartenstein, 1994). In transmission electron microscopy (EM), the pSJs are more easily identifiable than the sSJ as they are not obscured by electron dense material (Tepass and Hartenstein, 1994). In freeze fracture EM, the pSJs are distinguished from the sSJs by their rough appearance. Despite the difference in appearance, sSJs and pSJs function in the same way (Lane et al., 1994). The pleated septate
junctions are exclusively examined in this thesis and are referred to as the septate junctions from here out.

Besides maintaining the permeability barrier in glia, salivary glands and trachea (Tepass and Hartenstein, 1994; Lamb et al., 1998; Tepass et al., 2001), SJ components also regulate tracheal tube size and expanse of the apical domain (Paul et al., 2003; Llimargas et al., 2004), cardiac integrity (Yi et al., 2008), maintain epithelial polarity during embryogenesis (Laprise et al., 2009), movement of the epithelial sheet during dorsal closure and cell-cell adhesion (Fehon et al., 1994; Baumgartner et al., 1996; Lamb et al., 1998). Finally, cell division is localized to the SJ domain in columnar epithelia (Gibson et al., 2006), which points to another role for SJs beyond the formation of permeability barriers.

**The core septate junction complex**

The SJ proteins (Table 1.1; Table 1.2) analyzed in this thesis include three of the six core SJ proteins; Neurexin IV (NrxIV), Coracle (Cor) and Neuroglian (Nrg). The $\text{Na}^+/$$\text{K}^+$ pump $\alpha$ subunit ($\text{Atp}\alpha$) and $\beta$ subunit Nervana 2 (Nrv2) (Genova and Fehon, 2003; Paul et al., 2003), and Contactin (Cont) (Faivre-Sarrailh et al., 2004) (Figure 1.2C; Table 1.2) are also core SJ proteins and so are discussed briefly. Core septate junction proteins are defined as those that are necessary for formation of the SJs and a functioning permeability barrier. Mutant embryos for these genes no longer have septae to form the permeability barrier, as seen by the diffusion of fluorescently labeled dye across mutant epithelia (Lamb et al., 1998; Genova and Fehon, 2003; Paul et al., 2003) (Figure 1.2B; Figure 1.2A, Contactin not shown). They are also often necessary for localization of other core SJ components to the SJ domain (Genova and Fehon, 2003; Paul et al., 2003).
Neurexin IV, Coracle, Neuroglian and the Na+/K+ ATPase pump interact genetically and are dependent on each other for localization to the SJ domain (Ward et al., 1998; Ward et al., 2001; Genova and Fehon, 2003; Paul et al., 2003). Coracle, a homologue of mammalian protein 4.1, interacts with the cytoplasmic tail of transmembrane Neurexin IV, through the N-terminal FERM (protein 4.1, ezrin, radixin, moesin) domain. The cytoplasmic tail of Neurexin IV is similar to the cytoplasmic tail of Glycophorin C, which is known to interact with the vertebrate homologue of Coracle, protein 4.1, indicating a conserved interaction (Marfatia et al., 1994; Marfatia et al., 1995). Coracle and Neurexin IV are interdependent for localization to the membrane, so the downregulation or absence of one leads directly to the absence or downregulation of the other (Figure 1.2C, Table 1.2) (Bieber et al., 1989; Baumgartner et al., 1996; Ward et al., 1998).

Neuroglian is a transmembrane cell adhesion molecule (CAM) homologous to vertebrate Neurofascin-155 (Bieber et al., 1989; Charles et al., 2002). Homophilic binding activity through the extracellular domain is responsible for its cell adhesion properties (Hortsch et al., 1995) and Neuroglian may interact with NeurexinIV through its extracellular domain as well (Genova and Fehon, 2003). Neuroglian also links to the cytoskeleton by interacting through the cytoplasmic domain with Ankyrin (Figure 1.2C) (Dubreuil et al., 1996).

The Na+/K+ ATPase pump α and β subunits are also essential for SJ formation and maintaining the permeability barrier and form a complex with Neuroglian, Coracle and Neurexin IV (Genova and Fehon, 2003; Paul et al., 2003)(Figure 1.2C). The Na+/K+ ATPase pump is composed of two subunits, Atpα and the β subunit (Figure 1.2C), encoded by ATPα and Nrv2. The pump is necessary for the formation of the SJs and forms a complex with Nrg, NrxIV and Cor (Genova and Fehon, 2003; Paul et al., 2003). The Na+/K+ ATPase subunits bind to cytoskeletal elements and function as a scaffold for proteins essential for a paracellular barrier.
The Na\(^+\)/K\(^+\) ATPase pump function is not likely involved in SJ formation as mutations that inactivate the ATP\(\alpha\) catalytic subunit do not affect SJ formation or function (Paul et al., 2007). As the Na+/K+ ATPase pumps function at the SJ is not as well characterized as Nrg, Cor, and NrxIV, it was not analyzed in this thesis.

Contactin (Cont) (Faivre-Sarrailh et al., 2004) is another core septate junction protein that is necessary for formation and function of the permeability barrier. Contactin was identified in vertebrate paranodal SJs (a homologous structure to the Drosophila septate junction discussed later) where it interacts with the Neuroglian and Neurexin IV vertebrate homologues, Neurofascin 155 and Caspr/NCPI (Gennarini et al., 1989; Brummendorf and Rathjen, 1996). Even though Drosophila Contactin forms a complex with Neuroglian and Neurexin IV and is necessary for formation of the SJs, it is not necessary for localization of the core SJ components, and was therefore not analyzed in this thesis (Faivre-Sarrailh et al., 2004).

**Septate junction associated proteins**

A number of proteins are found localized to the SJ domain that are not part of the core group but have a moderating/indirect role, such as trafficking (Table 1.1). These are referred to here as SJ associated proteins. Some SJ associated proteins, such as Fascillin III, Lachesin (Llimargas et al., 2004) and Moody (Bainton et al., 2005; Schwabe et al., 2005) localize to the SJs but are not part of the core SJ complex.

The claudins Megatrachea (Behr et al., 2003), Sinuous (Wu et al., 2004), and Kune-kune (Nelson et al., 2010) (Figure 1.3A) can be considered integral SJ proteins, and Kune-Kune may qualify as another core SJ protein, depending on whether the septae are disrupted. The claudins are the only functional protein group shared between the vertebrate TJs and the Drosophila SJs
(Furuse et al., 1998; Tsukita and Furuse, 2002) (Figure 1.3A), and are necessary for SJ assembly in tube forming epithelia such as the trachea. The disruption of the claudins in the trachea leads loss of the SJ permeability barrier, but also to overgrowth of the apical domain, and an increase in tracheal tube size (Wu and Beitel, 2004; Wang et al., 2006). These last two phenotypes are examples of effects that extend beyond preventing diffusion, and set precedence for the SJs having roles outside establishing the permeability barrier (Behr et al., 2003; Wu and Beitel, 2004) (Figure 1.3B). What effect disruption of the claudins has on the SJs in columnar epithelia is not known (Wu and Beitel, 2004).

The next SJ associated group of proteins discussed is implicated in trafficking to the SJ domain. The Ly6 superfamily codes for different cell surface proteins and secreted ligands and contain an extracellular motif called a Ly6 domain, or three-finger motif. The Ly6 superfamily of proteins has been implicated in trafficking SJ components to the membrane. Boudin, crooked, coiled and crimpled are Ly6 proteins necessary for septate junction organization and trafficking NrxIV to the membrane (Hijazi et al., 2009; Nilton et al., 2010). The requirement of these four Ly6 proteins in SJ formation suggests members of this conserved family cooperate in SJ assembly through targeted vesicle trafficking to the membrane (Nilton et al., 2010).

Another group of SJ proteins discussed are those involved in establishing apical/basal polarity, including the scaffolding of proteins complexes to the SJs. Discs large (Dlg) is the prototypic membrane associated guanylate kinase (MAGUK) homologue originally identified as a tumour suppressor and then found to localize to and label the SJs (Noirot-Timothee and Noirot, 1980; Woods and Bryant, 1991). During embryogenesis, the Dlg complex (Dlg, Scribble and
lethal giant larvae) restricts the apical adherens junctions and establishes the basolateral SJs (Woods et al., 1996). Dlg\textsuperscript{M52} mutants survive to the larval stage, but produce overgrown imaginal wing discs. On closer inspection, these discs show an abundance of adherens junctions and gap junction, but are missing the septate junctions (Woods et al., 1996; Bilder and Perrimon, 2000). The two other proteins (Scribble (Scrib) and lethal giant larvae (Lgl)) that associate in the Dlg complex also localize to the SJ domain and are necessary for SJ formation (Bilder et al., 2000; Humbert et al., 2003; Yamanaka and Ohno, 2008).

Another protein necessary for maintaining apical/basal polarity in Drosophila, that also localizes to the SJ domain, is the FERM domain protein Yurt (Yrt) (Laprise et al., 2009). Yurt differs from the Dlg complex proteins in that it maintains epithelial polarity during organogenesis, but not during early embryogenesis, like Dlg, or in terminally differentiated tissue (Laprise et al., 2009). Yrt also differs in that it interacts with the core SJ proteins Cora, NrxIV, and Na\textsuperscript{+}/K\textsuperscript{+}ATPase to maintain apical/basal polarity (Laprise et al., 2009). Although these polarity proteins associate with the SJs, their roles outside of establishing and maintaining polarity are relatively unknown, as are their effect on the SJs past embryogenesis.

The last SJ associated protein discussed is Gliotactin, a tricellular septate junction associated protein necessary for the formation of the septae and permeability barrier (Figure 1.2C). Along with the core SJ protein NrxIV, Gliotactin is a main focus of study in this thesis. We discuss Gliotactin later as a component of the tricellular junction (TCJ) (Auld et al., 1995; Schulte et al., 2003).
Vertebrate permeability barriers: tight junctions and paranodal septate junctions

There are two types of permeability barriers in vertebrates that can be compared to the Drosophila SJs; the tight junctions (TJs) and paranodal septate junctions (PSJs). Both vertebrate junctions form permeability barriers and are functionally analogous to the SJs, but differ in their structure and tissue specificity (Tepass et al., 2001; Hortsch and Margolis, 2003).

The PSJs are structurally and functionally more analogous to the Drosophila SJs than the TJs (Einheber et al., 1997; Menegoz et al., 1997; Poliak et al., 2001; Tepass et al., 2001). Although both vertebrate junctions share homologous proteins with the Drosophila SJs, the homologues of the core Drosophila SJ proteins localize to the vertebrate PSJ and are necessary for forming that permeability barrier. These are Neurofascin-155, Caspr/Paranodin/NCPI, Protein 4.1B and Contactin, the homologues to the core septate junction proteins Neuroglian, Neurexin IV, Coracle and Contactin respectively (Einheber et al., 1997; Menegoz et al., 1997; Poliak et al., 2001; Tepass et al., 2001). Only the vertebrate claudins and Scribble localize to the TJs. Both vertebrate junctions share some evolutionary homology with the Drosophila SJs, but the PSJs offer a better structural comparison due to the shared core complex proteins.

Although the PSJs are structurally more analogous to Drosophila SJs, the vertebrate TJs form the permeability barriers in polarized epithelia, and like the SJs maintain apical/basal polarity (Izumi et al., 1998; Tsukita et al., 2001; Tsukita and Furuse, 2002). As this thesis investigates the role of SJs in columnar epithelia, the TJs are arguably more likely to share these functions in vertebrate epithelia than the PSJs. This is why the following chapters use the TJs, instead of the PSJs, as the vertebrate model when comparing and contrasting cell division in polarized epithelia between vertebrate and Drosophila cells.
Although SJs and TJs both form permeability barriers in columnar epithelia, they differ in appearance, localization, and composition. The TJs resemble fusion points, sometimes termed “kissing points” (Tsukita et al., 2001), between opposing membranes (Farquhar and Palade, 1965). In contrast, the SJs appear as a ladder-like array running basolaterally down the membrane (Tepass and Hartenstein, 1994). TJs localize apical to the vertebrate adherens junctions, opposite to the arrangement seen in Drosophila epithelia (Figure 1.2A) (Schneeberger and Lynch, 1992; Lamb et al., 1998; Knust and Bossinger, 2002; Genova and Fehon, 2003). This is an important difference to consider when comparing the localization of SJ and TJ homologues, as in some cases the localization of proteins is dependent on the membrane domain and others the junction type.

Essential TJ proteins include the occludins, claudins, junctional adhesion molecules (JAMS), and adaptor protein ZO-1. The occludins and claudins form the molecular basis for TJ strands and both have four membrane-spanning domains and two extracellular loops (Figure 1.4B) (Tsukita et al., 2001). Occludin is the first integral membrane protein localized to the TJs (Furuse et al., 1993; Ando-Akatsuka et al., 1996), but it is not necessary for the formation of the TJ strands (Ikenouchi et al., 2008). It is the claudins that form the backbone of the TJs and are necessary for their formation and function (Furuse et al., 1998; Tsukita et al., 2001). Ectopic expression of claudins is sufficient to induce formation of TJ strands in fibroblasts cells (Furuse et al., 1998). The junctional adhesion molecules (JAMs) are members of the CD2 subgroup of adhesion receptor immunoglobulin (Ig) superfamily, and are characterized by two Ig-like domains (Williams and Barclay, 1988; Barclay and Brown, 1997). They associate with intracellular scaffolding proteins and are involved in regulating apical/basal polarity through mediating tight junction formation (Ebnet et al., 2004). Unlike the claudins, occludins and JAMs
cannot induce TJ formation (Itoh et al., 2001) and disruption does not affect the permeability barrier.

The Drosophila claudins, Megatrachea, Kune-Kune, and Sinuous, are the only essential TJ protein homologues involved in Drosophila SJ assembly and function (Behr et al., 2003; Wu et al., 2004; Nelson et al., 2010). ZO-1 is a MAGUK/PDZ domain binding protein that binds claudin through its PDZ binding motif, allowing both proteins interact at the membrane (Itoh et al., 1999; Tsukita et al., 2001; Hamazaki et al., 2002). The closest Drosophila homologue to ZO-1 is the PDZ domain protein, Polycheatoid (Pyd), which localises to the adherens junctions and basolateral membrane (Chen et al., 1996; Takahisa et al., 1996; Wei and Ellis, 2001), but is not associated with the SJs. There are no Drosophila homologues for Occludin or the JAMs.

The tricellular tight junction (tTJ) and tricellular septate junction (TCJ)

As TJs and SJs both form bicellular seals between two opposing membranes, a permeability barrier gap is left at tricellular corners. At these corners, in both vertebrate and Drosophila epithelia, a tricellular junction forms to prevent the flow of solutes through the gap. These junctions are the Drosophila tricellular junction (TCJ) (Fristrom, 1982) and the vertebrate tricellular tight junction (tTJ) (Staehelin, 1973). In Drosophila, the TCJ is formed at the convergence of SJs from the neighbouring epithelial cells (Figure 1.1A), and in vertebrates, the tricellular tight junction (tTJ) forms at the convergence of three tight junctions (Figure 1.4A).

Both SJs and TJs descend vertically at tricellular corners. The tTJ forms a central tube through the meeting of adjacent bicellular TJs (Staehelin et al., 1969a; Staehelin et al., 1969b; Friend and Gilula, 1972; Staehelin, 1973; Wade and Karnovsky, 1974; Walker et al., 1985;
At the Drosophila TCJ, the SJ strands turn 90 degrees and run in an apical-basal direction down the cell (Figure 1.1A) (Fristrom, 1982; Noirot-Timothee et al., 1982; Walker et al., 1985). At these corners are “Plugs” that appear to attach to the descending SJ strands and anchor them in place (Figure 1.1A, B, D) (Fristrom, 1982). The TCJ permeability barrier is created by a series of plugs or diaphragms that span the length of the TCJ (Figure 1.1 B, D). Presumably the TCJ plugs are held in place through interactions with the descending septa from the SJ (Fristrom, 1982; Noirot-Timothee et al., 1982).

Although these tricellular structures differ in their ultrastructure, they both successfully form permeability barriers at the tricellular gaps. Looking at the vertebrate tTJ and associated proteins illustrates ways in which the Drosophila TCJ may function and interact with the SJs.

**Gliotactin and Tricellulin**

A shared feature of the tTJ and TCJ is the presence of designated tricellular junction proteins, necessary for the formation of both the tTJ and TCJ, and the permeability barrier. These are the vertebrate tTJ protein Tricellulin, and the Drosophila TCJ protein, Gliotactin.

A major question posed in this thesis is what role Gliotactin has in columnar epithelia, where it is the only protein known to localize exclusively to the TCJ (Figure 1.5A) (Schulte et al., 2003; Auld et al., 1995). Gliotactin is a transmembrane protein necessary for maintaining permeability barriers in a range of epithelia, including glia (Auld et al., 1995; Schulte et al., 2003). This effect is best illustrated in the salivary glands of homozygous mutant embryos. In wildtype embryos, injected dye is excluded from the lumen of the salivary gland by the SJ permeability barrier. In Gliotactin mutant embryos, the permeability barrier is no longer functional and dye diffuses across the membrane and into the lumen (Figure 1.5D). In these
mutant embryos, the SJs are more widely spaced and in some cases are missing between the opposing membranes (Figure 1.5C) (Schulte et al., 2003).

Drosophila Gliotactin is related to the vertebrate Neuroligins (Gilbert et al., 2001; Gilbert and Auld, 2005); all members of this family contain a highly conserved extracellular serine esterase-like domain (Figure 1.5B) that lacks one of three residues necessary for enzymatic function. Similar to Neuroligans, Gliotactin may form dimers or oligomers through its extracellular domain (Venema et al., 2004; Gilbert and Auld, 2005). It also has an intracellular domain with multiple predicted phosphorylation sites and an intracellular PDZ binding motif (Figure 1.5B). Gliotactin interacts genetically with the core SJ proteins (Venema et al., 2004) and the SJ associated MAGUK protein Discs large. Both Gliotactin and Discs large interact in a complex that is dependent on the Gliotactin PDZ binding motif (Figure 1.5B) (Schulte et al., 2006). Disc large also appears to be concentrated with Gliotactin at the TCJ (Woods et al., 1996; Schulte et al., 2006), suggesting that their biochemical interaction is related to function.

Though Tricellulin is the only exclusive tTJ associating protein in vertebrates, it is not a homologue of Gliotactin. This illustrates how the vertebrate TJs and Drosophila TCJ have evolved divergent but architecturally similar solutions to block the permeability barrier gap at tricellular corners. Tricellulin (TRIC) is a four-pass transmembrane protein related to the tTJ protein occludin. It has a tetra-spanning MARVEL (MAL and related proteins for vesicle trafficking and membrane link) domain that is associated with proteins involved in membrane apposition (Figure 1.4B) (Sanchez-Pulido et al., 2002). It is localized exclusively to the vertebrate tTJs (Figure 1.4C) and is maintained there by Occludin (Ikenouchi et al., 2008).

A question addressed in this thesis is whether a disruption of Gliotactin at the TCJ in columnar epithelia affects the maintenance of the SJs or the localization of SJ associated proteins.
Mutations for Gliotactin in the embryo suggest this is the case, as the SJs and Core SJ proteins are mislocalized basolaterally (Schulte et al. 2003). Downregulation of Tricellulin disrupts the permeability barrier and TJ organization (McCarthy et al., 1996; Ikenouchi et al., 2005; Yu et al., 2005). If Gliotactin is comparable to Tricellulin in its architectural role at the tTJ, then Gliotactin should have a similar role in maintaining the SJs and SJ proteins.

Although Tricellulin is the only known tTJ protein, it is the best vertebrate comparison for TCJ Gliotactin. However, Tricellulin mutations do not cause all known TJ permeability barrier phenotypes. This suggests that there are other TJ proteins to be discovered that may function more closely to Gliotactin at the tTJ (Riazuddin et al., 2006; Chishti et al., 2008).

**Gliotactin mutant phenotypes**

The permeability barrier defects seen in Gliotactin mutant embryos suggest that Gliotactin is necessary for the maturation, compaction and maintenance of the SJs (Figure 1.1B) (Schulte et al., 2003; Schulte et al., 2006). However, somatic clones for Gli$^{dv3}$, a null allele for Gliotactin, in the wing imaginal disc suggest that this function is not conserved through development. These clones are small (Schulte et al., 2006), do not survive to adulthood (Venema et al., 2004), and most importantly show no mislocalization of the SJ proteins NrxIV, Cora, and Dlg. This indicates that in the columnar epithelia of the imaginal wing disc, Gliotactin is not maintaining the SJs, and that once the SJs are established in epithelia, Gliotactin is no longer necessary for their maintenance (Schulte et al., 2006). This leaves the possibility that Gliotactin null clones undergo apoptosis, have a defect in cell division, or a combination of the two, possibilities investigated in this thesis.
Clones for the SJ core proteins NrxIV, Cora and Nrg in the wing imaginal disc show a similar phenotype to Gliotactin null clones, where clones do not reach a large size and do not survive to adulthood (Genova and Fehon, 2003). Whether Gliotactin is mislocalized in SJ mutant cells in the wing imaginal disc is addressed in this thesis, and also whether the similar phenotypes seen between Gliotactin and SJ mutant clones in the columnar epithelia are due to the mislocalization of Gliotactin.

**Drosophila SJ function beyond the permeability barrier**

Do SJs and Gliotactin have functions in columnar epithelial cells beyond a role in maintaining a permeability barrier and could this account for the somatic clone phenotypes? In the trachea the SJs are necessary for specifying the apical domain. When the SJs are disrupted, the apical domain expands and increases tracheal tube size, but without an accompanying loss of apical/basal polarity (reviewed in (Wu and Beitel, 2004). The SJ associated protein Yurt establishes apical/basal polarity during embryogenesis (Tepass and Hartenstein, 1994). These examples set precedence for the core SJ proteins and Gliotactin having roles outside maintaining a permeability barrier in columnar epithelia of the imaginal wing disc. One possible role for the SJs and Gliotactin is in cell division, as cell division occurs at the level of the SJs (Gibson et al., 2006). This thesis investigates whether the SJs and Gliotactin are necessary for cell division in the columnar epithelia and whether this is linked to clone size and lethality.

**Epithelia maintain junctions during cell division**

Architecturally complex cells are those that have a series of discreet junctional domains which include the adherens, septate, and gap junctions. They appear later in embryogenesis and
continue to undergo cell division in the imaginal disc until the late larval stages. At the start of mitosis in imaginal disc epithelia, the nucleus transitions from the basal side of the cell to the level of the SJs domain (Gibson et al., 2006). In Drosophila, the nucleus remains at this location throughout mitosis so that the plane of cell division is localized entirely at the SJs (Gibson et al., 2006).

An interesting aspect of the TJs and SJs in both vertebrate and Drosophila epithelium is that they are maintained even through cell division and the establishment of a new bicellular membrane (Baker and Garrod, 1993; Gibson et al., 2006). This raises the question of how and why cells maintain their junctional domains while at the same time building new junctions and cell-cell contacts. It is possible that there is a general theme of accommodating the junctions during cytokinesis in both vertebrate and invertebrate epithelia, and that the maintenance of polarity through cell division may facilitate rapid reformation of the polarized membrane (Reinsch and Karsenti, 1994), as well as maintaining the permeability barrier.

In Drosophila, cell division has been extensively studied in the developing embryo and in S2 cell culture. In these cell types the dynamic changes seen with markers for the contractile ring, spindles, midbody, midline furrow and centrosomes etc, have been mapped out for each corresponding stage (Straight and Field, 2000; Glotzer, 2001; Glotzer, 2003; Eggert et al., 2006). However, symmetric cell division has classically been studied in cells that have no septate junctions. Architecturally complex cells, such as the columnar epithelia containing SJs, have been excluded from cell division studies. Thus it is not know how cell division is modified in a cell with junction domains, or how the junctions are adapted to accommodate cell division. This thesis attempts to in part address this question through an analysis of all stages of cell division in wing disc epithelia.
Mitosis and Cytokinesis in Drosophila

In order to analyze cell division in Drosophila epithelia, a basic comprehension of the stages, events, and structures that allow mitosis to progress through to cytokinesis is required, along with an understanding of how cell division machinery interacts with the cell cortex. Cell division is examined first in architecturally simple S2 cells, and then in incrementally more complex cells, to illustrate what is already known about accommodating junctions during cell division.

Mitosis

The cell cycle can be divided into two alternating stages, interphase and the mitotic phase. Mitosis is characterized by the progression through a set of stages; prophase, prometaphase, metaphase, anaphase and telophase.

During prophase the chromosomes condense and a pair of centrosomes assembles adjacent to the nucleus with a pair of centrioles at their center. The centrosomes partially act as a coordinating centre for the microtubule spindle and each possesses a centriole (D'Avino et al., 2005) (Figure 1.6). Although the centrioles and centrosomes are involved in organizing the spindle, they are not essential in Drosophila epithelia (D'Avino et al., 2005; Basto et al., 2006).

Prometaphase is a transition phase between prophase and metaphase. During prometaphase, the nuclear envelope dissolves and the spindle microtubules extend from the centrosome poles to contact the kinetochore, the microtubules hook each chromatid, as well as non-kinetochore (aster) microtubules from the opposite pole of the forming spindle (D'Avino et al., 2005; Goshima and Scholey, 2010). Prometaphase is often considered part of metaphase and the remainder of this study does the same (Figure 1.6).
At metaphase, the mitotic spindle is fully formed and the chromosomes have lined up along the equatorial plate, equidistant from the two centroseome poles. When all the chromatid kinetochores have an attached microtubule bundle, anaphase begins. Two events are necessary for anaphase to proceed; the chromatids must be pulled apart and the spindle must elongate. The cohesion complex linking the sister chromatids is cleaved and the chromatids are pulled towards the centrosomes by shortening microtubules. The spindle is elongated through the non-kinetochore attached microtubules pushing the centrosomes to opposite ends of the cell. The separation of the chromatids occurs during early anaphase and the expansion of the spindle occurs during late anaphase. The central spindle also forms during late anaphase, spanning the distance between the separating chromosomes. For the purpose of this study we refer to both early and late anaphase as anaphase (Figure 1.6) (D'Avino et al., 2005; Goshima and Scholey, 2010).

At the onset of telophase, the chromosomes decondense and nuclei reform their nuclear membranes. At this point mitosis is complete; however, cell division is not. Telophase signifies the completion of mitosis and the onset of cytokinesis (Figure 1.6), described in detail below (D'Avino et al., 2005).

**Cytokinesis**

Cytokinesis is a separate process from mitosis and is necessary for the completion of cell division. It consists of 4 distinct, temporally controlled phases: formation of the cleavage furrow, membrane ingression, midbody formation and abscission (Figure 1.8) (Glotzer, 2005; Eggert et al., 2006). Much of what is known about Drosophila cell division comes from studies in S2 cell culture (Straight and Field, 2000; Glotzer, 2001; Glotzer, 2003; Eggert et al., 2006).
The Drosophila S2 cell culture line is derived from late stage, 20-24 hour embryos (Schneider, 1972). S2 cells do not adhere to each other, form junctions, or exhibit complex architecture, so they show the basic mechanisms of cytokinesis in a simple two dimensional plane, but not with respect to complex junctional domains like those present in columnar epithelia.

The steps and structural components central to the completion of cytokinesis are introduced with respect to an architecturally simple cell model, but an emphasis is placed on how they are positioned in relation to the cell cortex, as those are the aspects that particularly pertain to the organization of cytokinesis in architecturally complex columnar epithelia. Once the placement and organization of these events are introduced, what is known about their organization in incrementally more complex cells is discussed.

**Position of the cleavage plane is determined by the spindle**

Cytokinesis starts between late anaphase and early telophase when the cleavage plane is established at the equatorial cortex of the membrane, in line with the central spindle microtubules. Formation of the furrow and its position within the cell cortex is dependent on the position of mitotic spindle (Burgess and Chang, 2005; D'Avino et al., 2005). Ectopic spindles can induce ectopic cleavage furrows, demonstrating that a spindle is sufficient for cleavage furrow assembly (Rappaport, 1996; Rappaport, 1997; Glotzer, 2001; D'Avino et al., 2005). The mitotic spindle induces the cleavage plane through a combination of inhibitory and stimulatory effects directed at the cell cortex (Glotzer, 2001; Glotzer, 2004; D'Avino et al., 2005; Motegi et al., 2006) (Rappaport, 1986; Giansanti et al., 1998; Gatti et al., 2000; Bringmann and Hyman, 2005; D'Avino et al., 2005; Eggert et al., 2006); the astral microtubules (Figure 1.7A, light green) provide an inhibitory signal that prevents contraction, where as the central spindle
microtubules (Figure 1.7A, dark green), provide the cell cortex with a positive signal that specifies the cleavage plane and determines where the cell cortex will contract (Figure 1.7A, blue arrows) (Bringmann and Hyman, 2005; D'Avino et al., 2005).

The central spindle forms as bundled, inter-polar microtubules that are released from the spindle poles and localize between the separating chromosomes where they are maintained throughout cytokinesis (Figure 1.7B, right) (Shu et al., 1995). Proteins that interact with the spindle microtubules and cell cortex to facilitate formation of the cleavage furrow include a include centralspindlin, a highly conserved complex that is also required for assembly of the central spindle (D'Avino et al., 2005). The centralspindlin complex is composed of two components; a motor protein, MKLP1 sub-family Kinesin (Pavarotti); and a Rho-family (Rac) GTPase activating protein (RacGAP50C) (Mishima et al., 2002; Somers and Saint, 2003; D'Avino et al., 2005). In all organisms Pavarotti and RacGAP50C are necessary for assembly of the central spindle, furrow induction and subsequently completion of cytokinesis (Figure 1.7C) (Mishima et al., 2002) (Adams et al., 1998; Powers et al., 1998; Raich et al., 1998; Kuriyama et al., 2002; Matuliene and Kuriyama, 2002; Mishima et al., 2002; Somers and Saint, 2003).

Recruitment of the centralspindlin complex to the central spindle requires the conserved Aurora B complex (INCENP and Aurora B kinase), and Polo kinase (Figure 1.7C) (Lee et al., 1995; Adams et al., 1998; Adams et al., 2000; Kaitna et al., 2000; Adams et al., 2001a; Adams et al., 2001b; Adams et al., 2001c; D'Avino et al., 2005). For instance, the stable localization of Pavarotti to centralspindlin is dependent on Polo kinase and the Aurora B. A disruption of either leads to failure of the central spindle to form and results in disruption of cytokinesis (Terada et al., 1998; Giet and Glover, 2001).
The orientation of the spindle and proper localization of centralspindlin are necessary for the specification of the cleavage plane. How this is accomplished in columnar epithelia, where the cleavage plane is localized to the SJ domain, is unknown. It may be that the SJs themselves play a role in organizing the spindle and centralspindlin or their recruitment factors Aurora B and Polo kinase. Whether the cleavage plane is established is assayed in Gliotactin and core SJ mutants in order to determine disruption of the SJs or TCJ affects the localization of the spindle or positioning of the cleavage plane.

**Establishing the cleavage furrow and furrow ingression**

The next step in cytokinesis can be divided into two parts; establishing the site of the cleavage furrow and furrow ingression, and centralspindlin is necessary for both steps. Establishing the site of the cleavage furrow involves making the membrane at the cell cortex permissive to contraction, and ingression requires that the actinomyosin ring be fused to the membrane at this site and able to contract. RacGAP50C specifies the cleavage furrow through the activation of Rho A GTPase and mediating the inhibition of Rac GTPase at the equatorial cortex (D'Avino et al., 2005). This shift in equilibrium Between Rho A and Rac allows the cleavage furrow to be contracted by the actomyosin ring (Eggert et al., 2006) (Figure 1.7A-B).

The contractile ring is an actomyosin structure that is fused to the membrane at the site of the cleavage furrow and provides the mechanical force necessary for furrow ingression. During cleavage furrow specification, Rho A leads to the recruitment and stabilization of the F-actin filaments and the contractile ring motor protein, myosin II, to the equatorial cortex through the activity of the downstream effector Citron kinase (Figure 1.7D; Figure 1.8A-D) (Eggert et al., 2006). A third component of the contractile ring, anillin, is recruited to the cleavage furrow by
Rho GEF. Anillin is an actin-myosin II-septin binding protein that links the myosin II motor protein to the structural actin ring scaffold (Field and Alberts, 1995; Prokopenko et al., 1999; Oegema et al., 2000; Paoletti and Chang, 2000; Straight et al., 2005), and also acts as a scaffold for Rho A and centralspindlin at the cleavage furrow (Figure 1.7D) (D'Avino et al., 2005).

Ingression begins downstream of the Rho A pathway, where Citron kinase leads to the activation of a myosin light chain kinase (MLCK). This in turns activates Myosin II, catalyzing the contraction of the ring and membrane.

**Midbody formation**

The contraction of the actomyosin ring and furrow ingression leads to the formation of the midbody (or intercellular bridge), the third step in cytokinesis. The midbody consists of tightly bundled, anti-parallel microtubules, and an assortment of microtubule associating proteins (MAPs) that connect the two daughter cells during cytokinesis. Although it is not fully understood what the midbody does during cytokinesis, it is a convergence site for events associated with abscission, including degradation of cell cycle regulators, cytoskeleton rearrangements, membrane trafficking, and plasma membrane remodeling. In particular, the midbody appears to be crucial for the targeting of vesicles to the site of abscission (Albertson et al., 2005; Pohl and Jentsch, 2008), and the targeting is an intrinsically asymmetric process, as some types of vesicles arrive from only one prospective daughter cell (Gromley et al., 2005).

The components that control midbody targeting, assembly, and localization are unknown (Pohl and Jentsch, 2008), as is whether the midbody is asymmetrically inherited in all cell types. It is also unknown where the midbody is localized during cytokinesis in columnar epithelia.
Abscission

The final step of cytokinesis is abscission, where the two sides of the ingressing cleavage furrow are resolved into two distinct membranes. There are three essential components of abscission; formation of the new membrane through vesicles trafficking, remodeling of the two opposing membranes of the cleavage furrow, and cleavage of the midbody. The targeting of new membrane to the cleavage furrow is done through the exocyst complexes, where as the SNARE complex (v-SNARE and t-SNARE/Syntaxin) is necessary for the fusion of these vesicles to the furrow membrane (Gromley et al., 2005; Eggert et al., 2006). It is unknown whether the SNAREs also promote targeting of vesicles, or if that role is exclusive to the exocysts, and it is also unknown how vesicle targeting and fusion is coordinated with the midbody microtubules (Eggert et al., 2006). A multifunctional, ubiquitin-conjugating protein, BRUCE, localizes to the midbody (Pohl and Jentsch, 2008) and may act as a regulating platform for the delivery of endosomes and secretory vesicles (Pohl and Jentsch, 2008; Gould and Lippincott-Schwartz, 2009). Cleavage of the midbody and membrane remodelling are even more poorly understood.

BRUCE may also have a role in facilitating assembly of the abscission machinery at the midbody and may act as a diffusion barrier that separates the daughter cells before abscission (Pohl and Jentsch, 2008; Gould and Lippincott-Schwartz, 2009), but this is speculative.

Columnar epithelia maintain their junctions through cell division. During abscission, cell junctions are maintained at the old membrane as well as formed at the new membrane. An obvious role for the SJs would be in facilitating either the targeting or scaffolding for the vesicles and/or abscision machinery. Although it was beyond the scope of this thesis to look at vesicle trafficking and the abscission machinery directly, SJ and Gliotactin RNAi mutants were assayed for completion of cytokinesis to determine if there is potential for a role in abscission.
These studies have defined how cytokinesis is controlled in S2 cells, starting with the coordinated interaction of the spindle with the cell cortex at the beginning of mitosis, which is responsible for the recruitment of factors necessary for the furrow to ingress and contractile ring to assemble. How cytokinesis is controlled and initiated in polarized epithelia has yet to be determined.

In cells with simple architecture, there are two ways that cytokinesis defects manifest and both result in multinucleate cells. Class one cytokinesis mutants result from a block in ingression, and class two cytokinesis mutants form a furrow that regresses late in cytokinesis (Straight et al., 2005). Failure to form an ingression furrow can arise two ways; failure of furrow assembly and/or failure to form or contract the ring (Straight et al., 2005). Mutation in Rho A, Myosin II, and actin are all class one cytokinesis mutants, due to their failure to assemble the contractile ring or specify the cleavage furrow (Mabuchi and Okuno, 1977; De Lozanne and Spudich, 1987; Knecht and Loomis, 1987; Prokopenko et al., 1999; Tatsumoto et al., 1999; Straight et al., 2003; Straight et al., 2005). Failure to maintain furrow ingression can result from a disruption in the contractile ring activity late in cytokinesis, or failure to introduce new membrane between daughter cells (Straight et al., 2005). An example of a class two cytokinesis mutant is anillin, which regulates the contractile ring activity of Myosin II late in cytokinesis after an ingression furrow has already formed (Straight et al., 2005). It is not known how the contractile ring is assembled and held in columnar epithelia, or how furrow ingression is coordinated with the membrane and SJs, or whether the SJs are involved.
Cell division in simple, undifferentiated epithelia

Although the general blueprint of cell division illustrated above is conserved amongst all eukaryotic cell types, different cell architecture necessitates accommodations to this process, and cell division becomes increasingly complex as cell architecture increases in complexity. In the first two examples, cell division is examined in simple, undifferentiated cells that have adherens junctions but no septate junctions. Before introducing cell division in cells with increasingly complex architecture, how apical basal polarity and junctions form in Drosophila epithelia is first discussed.

Development of apical basal polarity and columnar epithelia architecture

Epithelial cells can possess a variety of complex junctions (including septate, adherens and gap junctions) that are restricted to their respective domains along the cell membrane. These various junctional domains are established during polarization of the epithelia and are representative of an epithelial cell’s apical/basal polarity (Figure 1.9A-B).

The adherens junctions are the first to appear and define the apical junctional domain of a polarized epithelial cell. They are established during early embryogenesis and are easily visualized. The adherens junctions are followed by the basolaterally localized septate junctions, and the last junctions established are the gap junctions. Although they do show some overlap with the septate junction domain they are typically localized basally in Drosophila epithelia (Fristrom, 1982).

In Drosophila epithelia, there are three complexes that specify apical/basal polarity in epithelia and establishing the apical adherens junctions and basolateral septate junctions (Figure
They are, in order of effect, the Bazooka/Par3 complex, the Crumbs complex, and the Lethal giant larvae group (Gibson and Perrimon, 2003).

The Bazooka/Par3 complex consists of Bazooka/Par3 (Baz), Par6, and αPKC. These three proteins associate with the sub-apical region and the apical plasma membrane where they regulate early phases of zonula adherens assembly (Figure 1.9A-B). The second apicalizing complex is the Crumbs complex (Stardust (Sdt), Discs lost (Dlt) and Crumbs (Crb)). The Crumbs complex also localizes to the sub apical region (Figure 1.9A-B) and is a crucial apical determinant. It acts later then the Bazooka/Par3 complex and is involved in adherens junctions maturation and stabilization (Tepass, 1996; Bachmann et al., 2001; Hong et al., 2001; Tepass et al., 2001; Gibson and Perrimon, 2003). In vertebrate epithelia the Crumbs and Bazooka/Par 3 complexes are associated with the tight junctions, but are not associated with the SJs in columnar epithelia (Woods and Bryant, 1991; Lane et al., 1994; Izumi et al., 1998; Medina et al., 2002) This is an example of localization dependent positioning, and not junctional association.

The Lgl group consists of Lethal giant larvae (Lgl), Scribble (Scrib), and Discs large (Dlg). This group is localized to the basolateral region of the membrane and plays a slightly later role in restricting the apical components and directing formation of the septate junctions. An example of this is seen in Dlg null mutants where the adherens junctions spread basally and the SJs do not form (Woods et al., 1996). The Lgl group counteracts the apicalizing activity of both the Crumbs complex and the Bazooka/Par3 complex, although the precise mechanism of interaction is unresolved. One possibility is that Lgl binds Par6-αPKC independent of Bazooka/Par3, thereby antagonizing the formation or activity of the Bazooka/Par3 complex (Betschinger et al., 2003). This is seen in vertebrate epithelia, where vertebrate Lgl bind to and inactivates the Par6-αPKC (Plant et al., 2003; Yamanaka et al., 2003). In vertebrate epithelia, the TJs are localized
apical to the adherens junctions, whereas the SJs are found basal to the adherens junctions (Figure 1.9A). Regardless of the differences in positioning, much of establishing apical basal polarity is conserved between Drosophila and vertebrates (Gibson and Perrimon, 2003). The major differences, besides the localization of the junctions themselves, are that the Bazooka/Par3 and Crumbs complexes help to specify the tight junctions, not the adherens junctions, and that Lethal giant larvae acts independently of its complex to antagonize the Par6-αPKC complex (Figure 1.9A-B). This demonstrates that these complexes are functionally conserved with respect to their localization within the membrane, and indicates that their roles in organizing cells division are likely conserved as well.

**Cell division in embryonic epithelia**

After cellularization in the Drosophila syncitial blastoderm the apical domain, or zonula adherens (ZA), is formed. The adherens junctions are the first established in the embryonic epithelia at the ZA (Tepass, 2002). The major constituent of the adherens junctions in both flies and vertebrates is the cadherin–catenin complex (DE-cadherin, Armidillo/β-catenin, and α-catenin) which links the junction to the actin cytoskeleton and a circumferential and contractile actin-myosin bundle that provides structure and tension at the adherens junctions (Tepass, 2002).

During cytokinesis in the blastoderm, the steps of cell division discussed in the previous section are conserved, except cells must accommodate the established ZA. Dividing cells maintain their adherens junctions, but also show dynamic reorganization as Myosin II and actin dissociate from the circumferential band at the apical membrane and relocalize to the contractile ring. At the end of cytokinesis, the ZA must expand into the new plasma membrane between the
two emerging cells, and presumably form new junctions. At this point, actin and Myosin II reassociate with the ZA (Tepass, 2002).

A small Ras-related GTPase, RAP1, is important for ensuring symmetric distribution of the adherens junctions at the new bicellular membrane (Knox and Brown, 2002) through its association with Pyd/ ZO-1, the TJ associating protein. It is unknown whether RAP1 and Pyd are necessary for the organization of the adherens junctions at the end of cytokinesis later in development, or whether there is an involvement of the SJs (Knox and Brown, 2002; Tepass, 2002). SJ and Gliotactin RNAi mutants were assayed for localization of the adherens junctions to address whether the SJs may be necessary for the relocalization of the adherens junctions after cytokinesis in columnar epithelia.

**Asymmetric cell division**

Asymmetric cell division has an added level of complexity, where the plane of cell division is organized along a superimposed polarity that may or may not be related to apical/basal polarity. A crucial component of asymmetric cell division is the polarized localization of cortical cues that establish the position of the spindle and cleavage furrow (Betschinger and Knoblich, 2004).

These positioning cues may be involved in organizing the spindle and cleavage furrow in the SJ domain during symmetric cell division in columnar epithelia. The specific cortical cues necessary for Drosophila cells to undergo asymmetric cell division are discussed below for two cell types; the embryonic neuroepithelia, which do not have septate junctions, and the imaginal disc SOP’s, which posses both adherens junctions and septate junctions (Betschinger and Knoblich, 2004).
**Drosophila neuroepithelia**

Drosophila embryonic neuroepithelia give rise to neuroblasts through asymmetric cell division and, like the blastoderm epithelia, possess adherens junctions but not septate junctions. Neuroblasts delaminate from the polarized neuroepithelia layer in the ventral neuroectoderm and continue to divide asymmetrically along the apical/basal axis to produce larger apical neuroblasts and smaller basal ganglion mother cells (Betschinger and Knoblich, 2004). Asymmetric cell division occurs through cortical cues that direct the formation of an asymmetric spindle (Rappaport, 1971; Ahringer, 2003; Thery and Bornens, 2006; Thery et al., 2007), and in neuroblasts Insuteable (Insc) is one of these positioning cues. Insuteable is localized and maintained at the apical domain by Bazooka/Par3, and Partner of Insuteable (Pins). From the apical domain, Insuteable provides positional information that couples the orientation of the spindle to the basal localization of cell fate determinants, such as Numb and Prospero, Partner of Numb (Pon) and Miranda (Ikeshima-Kataoka et al., 1997; Shen et al., 1997; Lu et al., 1998).

Bazooka/Par3 and Pins are expressed and localized apically in both the neuroblasts and neuroepithelia, so what prevents neuroepithelia from undergoing asymmetric cell division? The adherens junctions are one structure that maintain the symmetric plane of cell division in neuroepithelia, as disruption of the adherens junctions leads to asymmetric cell division (Lu et al., 2001). It is possible then that the adherens junctions are providing cues that specify a symmetric cleavage furrow and/or spindle.

Discs large and Lgl are also involved in asymmetric cell division in neuroepithelia. Lgl is responsible for the localization of cell fate determinants to the basal cortex (Betschinger and Knoblich, 2004), and Dlg affects the through an interaction with Kinesin heavy chain 73 (Khc73) microtubule motor protein (Ahringer, 2005). Although the involvement of Lgl and Dlg
in asymmetric cell division suggests that the septate junctions could play a role, neuroepithelia
do not have septate junctions. For clues as to how the septate junctions may be incorporated into
asymmetric cell division, one needs to look at the mechanosensory organ precursors (SOPs) in
the wing imaginal disc.

Cell division in Drosophila imaginal disc epithelium

Asymmetric cell division

The proximal region of the wing disc develops many simple mechanosensory organs (SOP)
called macrochaetes (Tepass, 2002) and asymmetric cell division is necessary for generating
these SOPs. Although much of asymmetric cell division is conserved between the SOPs and
embryonic neuroblasts, there are some differences that arise from differing distributions of
proteins in the membrane and the presence of SJs (Le Borgne et al., 2002; Tepass, 2002).

Drosophila SOP-cells are specified in the epithelia of the third instar imaginal wing disc
which have both adherens junctions and SJs. Instead of using the apical/basal axis to direct the
asymmetric orientation of the spindle, like occurs in neuroblasts, the SOP precursors use an
anterior/posterior axis, also known as planar cell polarity (Lu et al., 1999; Bellaiche et al., 2001;
Roegiers et al., 2001; Bellaiche et al., 2004). In Drosophila, the key mediators that specify
planar cell polarity are the serpentine receptor Frizzled (Fz) and the transmembrane protein
Strabismus (Stbm). The SOP-cells inherit the posterior localization of Frizzled and anterior
localization of Strabismus from the disc epithelium. This establishes the two cortical domains
(Bellaiche et al., 2004), much like apical/basal polarity is inherited from the neuroepithelia in
neuroblasts.
Bazooka/Par3 and Pins are involved in asymmetric cell division in the SOPs as well, however, their activity and localization is different. Inscuteable is not expressed in SOP-cells (Bellaiche et al., 2001), so it is Strabismus (Stbm) that binds to Pins and recruits it to the anterior cortex during prophase; together, both proteins restrict Bazooka/Par3 to the opposite, posterior side of the cell (Schaefer et al., 2001; Bellaiche et al., 2004). This is opposite of what is seen in Neuroblast cells, where Inscuteable localizes Bazooka/Par3 and Pins to the apical cortex (Betschinger and Knoblich, 2004). Dlg is also involved in SOP asymmetric cell division, where it restricts Bazooka/Par3 to the posterior cortex, thereby maintaining asymmetric polarity (Bellaiche et al., 2001; Betschinger and Knoblich, 2004). Since Dlg is involved in maintaining the asymmetric axis in SOPs, it is possible that Dlg could also be involved in organizing the axis during symmetric cell division in the wing disc, and this question is addressed in this thesis.

**Symmetric cell division in wing disc epithelia**

Symmetric cell division in vertebrate and Drosophila epithelia is associated with the TJs and SJs (Reinsch and Karsenti, 1994; Gibson et al., 2006), however, it is not known how the spindle and plane of cell division are localized to the permeability barrier domains or whether the SJs or SJ associated proteins, such as Dlg, are necessary for cytokinesis. Although the SJ associated proteins Lgl and Dlg are involved in asymmetric cell division in neuroblasts and SOPs respectively (Betschinger and Knoblich, 2004), it is not known whether these functions are carried over to symmetric cell division in the disc. This thesis addresses these questions by determining whether a disruption in the SJs affects cytokinesis and the localization of cell division to the SJ domain.
In this study, Drosophila 3rd instar imaginal wing discs (Figure 1.10) are used to investigate symmetric cell division in an architecturally complex cell type. There are two cell types present in the third instar imaginal wing disc, the overlying peripodial cells (Figure 1.10C, arrow; Figure 1.10E) and the columnar epithelium (Figure 1.10C, arrowhead; Figure 1.10D). The peripodial cells are large, flat, squamous-like polarized epithelial cells that form the outer top layer of the bicellular imaginal wing disc, and cover the smaller diameter, tall columnar epithelia. Although I discuss the peripodial cells in relation to cell division briefly in Chapter 2, the primary cell type used in this thesis are the columnar epithelia.

The third instar columnar epithelia exhibit the complex architecture and apical/basal polarity necessary to address how architecturally complex polarized epithelia undergo cell division. The second criterion this tissue type meets is that the cells are undergoing continuous cell division.

The reasons for using the Drosophila model system over vertebrate cellular culture to study these types of junctions include the ease of genetic manipulation and the ability to express RNAi using the Gal4/UAS expression system (Figure 1.10B), a short lifecycle and the ability to produce large quantities of sample tissue in a relatively short time. Another advantage to using Drosophila tissue is that the cells can be genetically manipulated and studied in an otherwise wildtype background (Figure1.10B).

The Thesis Questions

This thesis aims to address two aspects of cell division in columnar epithelia; 1) how does cell division progress in dividing wildtype cells?; and 2) are the SJs and Gliotactin necessary for cell division to occur. The involvement of the SJs and Gliotactin is addressed from two different
perspectives; 1) Is the localization of the plane of cell division to the SJ domain dependent on the SJs or Gliotactin, and 2) are SJs and Gliotactin necessary for cell division to proceed? A summary of the research and questions asked in each thesis Chapter is presented below.

How does cell division proceed in architecturally complex epithelium of the Drosophila imaginal wing disc?

In Chapter two I ask how the stages and structures associated with cell division localize in columnar epithelia with respect to the SJ domain. I map all the key stages of cell division and cytokinesis to a physical location within the cell and provides a reference point for the structures with respect to the SJs, which are used as reference in chapters three and four.

The map of cell division starts by determining the location of the SJs at each stage of cell division, using GFP tagged fusion proteins for three SJ markers, Dlg-GFP, Nrx4-GFP, and Nrg-GFP. The cleavage furrow, ingression furrow, spindle, centrosomes, contractile ring, microtubule cytoskeleton, nuclei and the midbody are mapped in relation to the SJs as cells progress through cell division and into cytokinesis.

How do cells maintain the plane of cell division at the level of the septate junctions?

In Chapter three, I used RNAi knockdown against core SJ proteins and Gliotactin to address whether the SJs or Gliotactin are necessary for localizing the plane of cell division to the SJ domain. Downregulation of Gliotactin or the core SJ proteins all led to a basal mislocalization of the plane of cell division as assayed by the centrosomes and DNA. The change of the plane of cell division correlated with a loss or basal spread of Gliotactin, suggesting that Gliotactin is necessary to localize the plane of cell division to the SJ domain.
Finally, I argue that Gliotactin maintains the plane of cell division through stabilizing the microtubules. The cytoskeleton microtubules are necessary for nuclear migration as well as structure and support. Overall, studies in multiple organisms, including Drosophila, strongly implicate the microtubules and microtubule associated motor proteins in nuclear migration (Fan and Ready, 1997; Whited et al., 2004; Baye and Link, 2008).

**How do imaginal disc and columnar epithelial cells complete cytokinesis?**

In Chapter four I look at the effect of downregulation of the SJs and Gliotactin on cytokinesis. As cytokinesis is centered at the SJs, the goal of this chapter was to determine if disrupting the SJs would block any part of cytokinesis.

SJs were disrupted by expressing Nrx IV RNAi in the imaginal wing disc and cells were assayed for their ability to form the ingression furrow and contractile ring. Disruption of NrxIV leads to a failure of the ingression furrow to form properly and a disconnect between the contractile ring and the membrane, suggesting these cells do not complete cytokinesis.
Chapter 1 Figure Legends

Figure 1.1 Drosophila septate and tricellular junctions

A) The adherens junctions (yellow) surround the apical region of the cell in a band. This region is also referred to as the zonula adheren (ZA). Directly below the adherens junctions, the septate junctions are localized (red). The SJs appear as sets of strands that stretch basolaterally down the cell. Where three cells meet, the SJ strands turn 90 degrees, and with other SJ associated proteins form the tricellular junction (green). Both the TCJ and the SJ are necessary for the permeability barrier.

B) Formation of the tricellular junction. For each stage cells are shown at the TCJ to show both paracellular and cytoplasmic localization. At Stages 11 and 15 of embryogenesis, the septate junctions begin to form. During stage 11, the SJ and TCJ proteins are dispersed through the membrane. During stage 13, the SJ strands begin to assemble and localize to the basolateral domain (black). The TCJ also starts to assemble (yellow), as well as the plug (orange). Through to stage 15, the SJs compact apically and the permeability barrier forms. The maturation of the TCJ (Orange) through Gli (red) accomplish this compaction (adapted with permission from Schulte et al., 2003).

C) Freeze fracture SEM of Drosophila epithelia. Arrows indicate the septate junction strands (SJ) at the tricellular junction (TCJ) run 90° to the membrane. D) Cartoon indicating the regions shown in C. The SJ strands run basolaterally where three cells meet, and a plug forms at the TCJ (arrow) (adapted with permission from Noirot-Timothee et al. 1982).
Figure 1.2 The permeability barrier is disrupted in SJ and TCJ mutants.

A. Confocal sections of live, late stage embryonic salivary glands. Embryos were injected with fluorescently labeled dextran. In wildtype embryos the dextran is prevented from entering the salivary gland lumen by the SJ permeability barrier (arrow). In a septate junction mutant, (Nrg-), the permeability barrier is absent and dye fills the lumen (arrow). In a TCJ mutant (Gli-), the permeability barrier is also absent and the salivary gland lumen fills with dextran (arrow). Scale bar represents 25 μm (adapted with permission from Genova et al. 2003).

B. TEM of embryonic epithelia mutant for the core complex of SJs (cor, Nrv2, ATPα, Nrg) and TCJ Gliotactin (Gli). i shows a wildtype epithelia with the SJs (arrows) and adherens junctions (arrowheads). In the SJ mutants (ii-vi), the septae are absent. In all cases the adherens junctions are unaffected (adapted with permission from Genova et al. 2003).

C. A cartoon diagram illustrating some of the SJ components shows potential interactions between SJ components. It includes the core complex and SJ associated proteins such as Scribbled and Dlg. The legend shows the various binding domains. Physical interactions between Coracle, Neurexin IV, Neuroglian and Contactin have been demonstrated (see text). Broken arrows indicate postulated interactions between the PDZ binding motifs of Gliotactin and Neurexin IV and the PDZ domain of Discs large and Scribble. Coracle could also potentially bind to the 4.1 protein binding domain on Dlg. It is still unknown what proteins the extracellular domain of Gliotactin, the Na+/K+ ATPase pump and Fascilin III interacts with (adapted with permission from Hortsch and Margolis et al. 2003).
**Figure 1.3 Components of the SJs and their functions**

A. A comparison of Drosophila and vertebrate cell-cell junctions along the lateral membrane. This includes a partial listing of Drosophila SJ proteins and their vertebrate homologues. The claudins (Sinuous Megatrachea and Kune-kune) are the main group of Drosophila SJ proteins shared between the tight junctions and septate junctions. Drosophila Coracle is a member of the protein 4.1 superfamily, which also localizes to the vertebrate tight junction domain. Red is used to indicate marginal zone and tight junction associated proteins. Green indicates the adherens junctions and adherens junction associated proteins. Blue and black indicate the septate junctions, septate junction associated proteins and basolateral proteins. (adapted with permission from Wu and Beitel, 2004)

B. Two models for the increase in tracheal tube size seen when the SJs are disrupted. i. Apical specification model. Disruption of the SJs leads to an increase in apical specification (mislocalization of Dlg) leading to an increase in tube size. This is not through a loss of polarity and basal spread of the apical components. ii. Extracellular matrix model. Here the SJs are required for control and specification of the apical ECM. The apical ECM is necessary for specifying tube size by a yet unidentified mechanism (adapted from Wu and Beitel, 2004).
A

**Drosophila**

Marginal zone

Adherens junction

Septate junction (barrier junction)

Kinesin-kinesin

Sinuous (Snu)

Megatrachea (Mega)

DE-Cadherin (DE-Cad)

Armadillo (Arm)

Crumbs (Crb)

DaPKC (DαPKC)

DmPar-6 (DrpPar-6)

Stardust (Std)

Bazooka (Baz)

**Vertebrate**

Claudins

E-Cadherin

β-catenin

CRB1

aPKC

Par-6

Par1

ASIP/Par-3

Band 4.1

Tight junction (barrier junction)

Adherens junction

Basal region

B

**Apical specification model of tube-size control**

**Wild-type**

- Marginal zone
- Adherens junction
- Septate junction

**SJ mutant (Digg mislocalized)**

- Marginal zone
- Adherens junction
- Septate junction

**Increased apical specification**

**Increased tube size**

**Apical extracellular matrix model of tube-size control**

**Wild-type**

- Septate junction

**SJ mutant**

- Septate junction

**Increased tube size**
Figure 1.4 The tricellular tight junction

A) A schematic diagram showing the organization of the tTJ. The TJ strands turn 90 degrees and form a central tube. The tight junctions strands each contact a neighboring strand thereby maintain the permeability barrier.

B) Membrane folding models for mouse Tricellulin and Occludin. Both proteins share two structural characteristics besides their four transmembrane domains; 1. The first extracellular loops contain a high content of glycine and tyrosine, 2. The cytoplasmic COOH- terminal (red box).

C) Localization of Tricellulin in mouse Eph4 epithelial cells. Tricellulin (red) is localized to the tricellular corners, whereas occluding (green) is distributed along the tight junctions. The bottom right panel shows the vertical sections of the tight junctions (white arrows). Both Tricellulin and occluding are localized within this domain (panels A-C). Scale bar represents 10 µm (adapted with permission from Ikenouchi et al. 2005).
Figure 1.5 Gliotactin and the TCJ

A. Gliotactin is localized to the TCJ. i-iv) Drosophila epidermal cells (i-ii), salivary gland cells (iii), and peripodial cells (iv) stained with Gliotactin (Gli, green) and SJ proteins Dlg (red, i,iv) and Coracle (Cor, red, ii) and Neuroglian (Nrg, red, iii). Note the ribbon like structure Gliotactin forms in the peripodial cells (iv, arrow) (adapted with permission from Schulte et al., 2006)

B. Cartoon representation of Gliotactin domains and point mutations. Gliotactin consists of a 959 aa type 1 transmembrane protein with an extracellular signal sequence, an extracellular serine esterase-like domain and an intracellular PDZ binding domain. The location of known ethyl methanesulfonate (EMS) alleles are indicated (adapted with permission from Venema et al. 2004)

C. Mutations in Gliotactin lead to increase spacing and loss of the SJ septae. Transmission electron microscopy (TEM) of a wildtype stage 17 embryonic epithelia showing the TCJ. (Bar in i) = 100nm;  Bar in ii) = 50nm).

i) Clusters of septae (brackets) are interspersed with single or pairs of septae. ii shows a TCJ with the septae at the all three bicellular junctions (arrowheads). iii-v) In Gliotactin mutant embryos septae are sparse. Singles, pairs and clusters of septae are predominantly found (iii, iv, arrows). v. The septae are not found at all bicellular contacts of the TCJ (v, arrows) (adapted with permission from Schulte et al., 2003).

D. Live embryos are injected with 10kD rhodamine-dextran (red) conjugate to evaluate the integrity of the permeability barrier. The salivary gland membrane is marked with gapGFP (green). i-iii) In wildtype embryos rhodamine is excluded from the lumen of the salivary gland (asterisk) by the SJs. b indicates the basal side, and a indicates the apical side. Arrowhead and x indicate the point of rhodamine block and the basal region of the SJs. iv-vi) In Gli homozygous
mutant embryos, the lumen fills with rhodamine-dextran. iv, arrowheads). The SJ permeability barrier is disrupted (adapted with permission from Schulte et al., 2003).
Figure 1.6 Mitosis

The table shows the stages of mitosis from prophase to telophase and illustrates the main events that are associated with each stage. Beside each stage is a cartoon of each mitotic phase. The centrosomes (red), microtubules and nuclear envelope (black), chromosomes (blue) are shown.
<table>
<thead>
<tr>
<th>Stage</th>
<th>Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prophase/</td>
<td>Nuclear envelope dissolves</td>
</tr>
<tr>
<td>Prometaphase</td>
<td>Chromosomes condense</td>
</tr>
<tr>
<td></td>
<td>The microtubule spindles contact the kinetochores on the chromatids</td>
</tr>
<tr>
<td>Metaphase</td>
<td>Chromosomes line up at the equatorial plate</td>
</tr>
<tr>
<td></td>
<td>Spindle microtubules attach to either the kinetochores or the opposing non-kinetochore microtubules</td>
</tr>
<tr>
<td>Anaphase</td>
<td>The microtubules retract, separating the chromosomes.</td>
</tr>
<tr>
<td></td>
<td>Non-kinetochore associated microtubules extend. And the central spindle forms</td>
</tr>
<tr>
<td>Telophase</td>
<td>The chromosomes condense and the nuclear envelopes reform.</td>
</tr>
<tr>
<td></td>
<td>Telophase marks the end of mitosis and the start of cytokinesis, highlighted by the pinching of the cleavage or ingression furrow</td>
</tr>
</tbody>
</table>
Figure 1.7 The spindle establishes the cleavage plane

A. Cartoon depiction of the distribution of microtubules during anaphase (left) and the force they exert on the cell cortex to induce formation of the cleavage furrow (right). In anaphase, astral microtubules (light green) stretch from the centrosomes (black circles) to the equatorial cortex in a plus end direction. The central spindle (dark green) is composed of overlapping microtubules that extend between the chromosome masses (light blue) and the equatorial cortex. The red and blue arrows in the cartoon to the right indicate the effect the microtubules have on the cortex. Red arrows indicate polar relaxation signals from the astral microtubules, preventing contraction and cleavage furrow formation, and the blue arrows indicate cortex stimulation from the central spindle, allowing for formation of the cleavage furrow (adapted from Eggert et al. 2006)

B. Cleavage furrow formation. Cells depicting before cleavage furrow formation (left) and during cleavage furrow formation (right). An equilibrium between Rho and Rac at the cell cortex results in a rigid cell membrane/cytoskeleton that results in the cell rounding seen during early cell division/mitosis (left). A high concentration of Rho at the equatorial cortex is necessary for contractile ring formation (blue ring) and furrow ingression. The inset rectangles indicate the actomyosin filaments at the equatorial cortex. Note the anti-parallel alignment of the microtubules at the cell cortex (blue), during cleavage furrow formation (adapted with permission from D’Avino et al. 2005).

C. A diagram depicting the assembly of the centralspindlin complex and other associated components at the cleavage furrow. Centralspindlin is composed of a Kinesin family motor component, Pavarotti (green oval) and a Rac GAP protein, RacGAP50C (yellow rectangle). The centralspindlin RacGAP activates the RhoGEF, Pebble, which in turns activates RhoGTPase. RacGAP also positively affects the RhoA GTPase by inhibiting the activity of RacGTPase at the
equatorial cortex, allowing for the contraction of the actinomyosin filaments (adapted with permission from D’Avino et al. 2005).

D. The organization of the contractile ring and Rho pathway components at the ingression furrow. Anillin (red) is thought to act as a general scaffold between the actomyosin filaments (actin, brown; myosin, green+black), microtubules (blue), septin (purple), RhoA (green), centralspindilin (RacGAP, blue; Pavorotti/MKLP, pink), and Pebble/Rho GEF (yellow) (adapted with permission from D’Avino et al. 2008).
**Figure 1.8 Progression of the ingression furrow through cytokinesis**

Immunofluorescence of dividing cells (undefined) at metaphase, anaphase, early telophase and late telophase. The location of the cleavage furrow is marked with Anillin (red), the microtubules and spindle are shown in green, and DNA is shown in blue. To the left of each immunofluorescence image is a cartoon depiction.

A. Metaphase. Astral microtubules extend from the poles to contact the cortex microtubules and the chromosome kinetochores. The cleavage plane has not yet been established.

B. Anaphase. The astral spindles are released from the centrosomes and occupy the space between the separating chromosomes, forming the central spindle. The cleavage furrows has been specified and the contractile ring has been assembled (red). Arrow indicates where both contractile ring markers Anillin and Zipper/myosin II heavy chain are localized.

C. Telophase (early). The contractile ring has caused ingression of the furrow due to the activity of myosin II on f-actin. The central spindle is condensed into a region referred to as the midzone. MAPs such as Jupiter concentrate to this area.

D. Late telophase. The opposing furrows have almost fully ingressed. The central spindle has been compressed into the midbody, a microtubule dense structure that forms a bridge between the two daughter cells, and in Drosophila is also marked by the MAP Jupiter. Completion, or abscission, occurs when the intercellular midbody bridge is resolved. (adapted with permission from Eggert et al. 2006).
Figure 1.9 Establishing apical basal polarity

i and ii) respectively show invertebrate and vertebrate epithelia. The zonula adherens (adherens junctions, ZA) are red, the septate junctions (SJs) are blue. In the invertebrate model the sub-apical region (SAR) is green, where green is used to highlight to apical tight junctions (TJs) in vertebrate epithelia. Proteins shown in iii and iv are colour coded to their respective domains.

iii) In Drosophila epithelia Disc large (Dlg), Scribble (Scrib) and lethal giant larvae (Lgl) form a complex that acts to restrict apical determinants (Bazooka complex and Crumbs complex) from the basolateral domain. Lgl in particular has been linked to this role. The Crumbs complex Discs lost (Dlt), Crumbs (Crb) and Stardust (Sdt) is a necessary apical determinant. iv) In the vertebrate system, Lgl binds to and inactivates Par6/aPKC. When it is phosphorylated by aPKC, it releases aPKC and Par6 to bind Par3/Bazooka and form an active complex. They then act along with the PATJ, Crumbs and PALS1 complex to specify the vertebrate TJ domain (adapted with permission Gibson and Perrimon. 2003).
(i) SAR  
ZA  
SJ

(ii) TJ  
ZA

(iii) apical determinant
Baz  
Par6  
aPKC

Sat  
Crb

Dlg  
Scr

Apical inhibition

Insect (Drosophila)

(iv) PATJ  
PALS1

Par3-Baz

aPKC

Par6

Active complex

Par3-Baz

Par6  
Lgl

Inactive complex

Vertebrate

Current Opinion in Cell Biology
**Figure 1.10 The Drosophila 3rd instar imaginal wing disc**

A. 3rd instar imaginal wing disc. Khaki indicates the wing pouch. The anterior/posterior (blue dotted line) and dorsal/ventral (black dashed line) axes are indicated. The apterous dorsal region is indicated in red. Sensory organ precursors are indicated in the thorax region (solid line) as black dots.

B. A third instar imaginal wing disc immunolabeled for Dlg (green), and expressing mCD8-GFP (red) under the control of the apterous GAL4 driver.

C. 3rd instar imaginal disc wing pouch. green indicates Dlg and red indicated Gliotactin. Columnar epithelia are indicated by the white arrowhead and shown close up in D. Peripodial cells are indicated with the white arrow and shown close up in E. In E Dlg is red and Gli is green.
Table 1.1 Drosophila SJ proteins and functions

Shown are main septate junction proteins, their homologues and functions. TJ indicates if the vertebrate homologue is associated with the tight junctions. Question marks denote lack of a close homologue or undetermined (Horscht and Margolis. 2003).
<table>
<thead>
<tr>
<th>Drosophila Molecule</th>
<th>Vertebrate homologue</th>
<th>Gene family</th>
<th>Function in Drosophila</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuroglian</td>
<td>Neurofascin 155</td>
<td>L1 family CAM</td>
<td>SJ formation, core protein</td>
</tr>
<tr>
<td>NeurexinIV</td>
<td>Caspr/Paranodin</td>
<td>Neurexin family</td>
<td>SJ formation, core protein</td>
</tr>
<tr>
<td>Coracle</td>
<td>Protein 4.1</td>
<td>4.1 Proteins</td>
<td>SJ formation, core protein</td>
</tr>
<tr>
<td>Na⁺/K⁺ ATPase</td>
<td>Na⁺/K⁺ ATPase</td>
<td>SJ formation, core protein</td>
<td></td>
</tr>
<tr>
<td>Dcontinctin</td>
<td>Contactin</td>
<td>F11/Contactin</td>
<td>SJ formation, core protein</td>
</tr>
<tr>
<td>Gliotactin</td>
<td>Neuroligin</td>
<td>Neuroligins/electrotactins</td>
<td>TCJ protein, SJ compaction</td>
</tr>
<tr>
<td>Megaetrachea</td>
<td>Claudins (TJ)</td>
<td>Claudins</td>
<td>SJ formation, tracheal tube size</td>
</tr>
<tr>
<td>Sinuous</td>
<td>Claudins (TJ)</td>
<td>Claudins</td>
<td>SJ formation, tracheal tube size</td>
</tr>
<tr>
<td>Kune-kune</td>
<td>Claudins (TJ)</td>
<td>Claudins</td>
<td>SJ formation, tracheal tube size</td>
</tr>
<tr>
<td>Discslarge</td>
<td>hDLG/Sap97</td>
<td>MAGUK family</td>
<td>Establish apical/basal polarity and SJ domain</td>
</tr>
<tr>
<td>Scribble</td>
<td>hscrb1 (TJ)</td>
<td>Lap family</td>
<td>Establish apical/basal polarity and SJ domain</td>
</tr>
<tr>
<td>Lethal 2 giant larvae</td>
<td>hLgl1/lgl2</td>
<td>Lgl protein family</td>
<td>Establish apical/basal polarity and SJ domain</td>
</tr>
<tr>
<td>Varicose</td>
<td>PALS2</td>
<td>MAGUK (vari subgroup)</td>
<td>SJ formation, tracheal tube size</td>
</tr>
<tr>
<td>FasciclinIII</td>
<td>?</td>
<td>?</td>
<td>Cell adhesion/axon path finding</td>
</tr>
<tr>
<td>αSpectrin</td>
<td>αSpectrin</td>
<td>spectrin super family</td>
<td>mem. attachment to the actin cytoskeleton</td>
</tr>
<tr>
<td>Boudin</td>
<td>?</td>
<td>Ly6 super family</td>
<td>SJ organization (secreted)</td>
</tr>
<tr>
<td>Crooked</td>
<td>?</td>
<td>Ly6 super family</td>
<td>SJ organization/vesicle trafficking</td>
</tr>
<tr>
<td>Coiled</td>
<td>?</td>
<td>Ly6 super family</td>
<td>SJ organization/vesicle trafficking</td>
</tr>
<tr>
<td>cramped</td>
<td>?</td>
<td>Ly6 super family</td>
<td>SJ organization/vesicle trafficking</td>
</tr>
<tr>
<td>Lachesin</td>
<td>IgLONs</td>
<td>Ig superfamily(cam sub)</td>
<td>SJ organization/ homphilic cell adhesion</td>
</tr>
<tr>
<td>Moody</td>
<td>?</td>
<td>GPCR</td>
<td>Glial cell permeability barrier</td>
</tr>
</tbody>
</table>
Table 1.2 The Drosophila SJ and TCJ proteins

A list of the SJ proteins used in this study along with their abbreviations. TCJ Gliotactin is included. Proteins domains and localization are included, along with known binding partners. The effect each protein has on SJ formation and maintenance is also listed.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein domains</th>
<th>Protein localization</th>
<th>Binding partners</th>
<th>SJ formation</th>
<th>SJ compaction Required?</th>
<th>SJ maintenance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coracle (Cor)</td>
<td>FERM (protein 4.1, ezrin, radixin, moesin)</td>
<td>Cytosolic/SJs</td>
<td>Neurexin IV, associates with Nrg, Nrv2, Atpα in complex</td>
<td>Necessary, required for localization of NrxIV</td>
<td>no</td>
<td>Required/maintains TCJ</td>
</tr>
<tr>
<td>Neurexin IV (NrxIV)</td>
<td>EGF-Like domain, Lamin G domain, Lectin domain</td>
<td>Transmembrane/SJs</td>
<td>Coracle, associates with Nrg, Nrv2, Atpα in complex</td>
<td>Necessary, required for localization of Cora</td>
<td>no</td>
<td>Required/maintains TCJ</td>
</tr>
<tr>
<td>Neuroglian (Nrg)</td>
<td>Fibronectin type 3 domain, Immunoglobulin domain</td>
<td>Transmembrane/SJs</td>
<td>Nruooglian, Neurexin IV and Dcontactin extracellular domains?</td>
<td>Necessary</td>
<td>no</td>
<td>Required/maintains TCJ</td>
</tr>
<tr>
<td>Nervana 2 (Nrv2)</td>
<td>ATPase pump subunit</td>
<td>Transmembrane/β-Subunit</td>
<td>Atpα, Associates with NrxIV, Cora, Nrv2, ATPα in a complex</td>
<td>Necessary</td>
<td>no</td>
<td>Disruption leads to basal spread</td>
</tr>
<tr>
<td>ATPα</td>
<td>ATPase pump subunit</td>
<td>Transmembrane/α-Subunit</td>
<td>Nrv2, Associates with NrxIV, Cora, Nrv2, ATPα in a complex</td>
<td>Necessary</td>
<td>no</td>
<td>Disruption leads to basal spread</td>
</tr>
<tr>
<td>Gliotactin (Gli)</td>
<td>PDZ/ serine-colinesterase</td>
<td>Transmembrane/TCJ</td>
<td>Complex with Dlg/ Binding partners unknown</td>
<td>No</td>
<td>Necessary only for permeability barrier function</td>
<td></td>
</tr>
<tr>
<td>Discslarge (Dlg)</td>
<td>PDZ binding domain</td>
<td>Cytosolic/ SJ/TCJ</td>
<td>Scribble/ lethal giant larvae/ Complex with Gliotactin</td>
<td>Necessary for establishing apical/basal polarity and specifying SJ domain</td>
<td>no</td>
<td>Required for TCJ and Gliotactin</td>
</tr>
</tbody>
</table>
II. Cell division in polarized epithelia cells of the Drosophila imaginal wing disc

Introduction

In Drosophila, cell division has been extensively studied in the developing embryo and cultured S2 cells. In these cell types, the dynamic changes seen with a wide range of mitotic markers (contractile ring, mitotic spindle, midbody, midline furrow and centrosomes), have been mapped out for each corresponding stage (Straight and Field, 2000; Glotzer, 2001; Glotzer, 2003; Eggert et al., 2006). However, S2 cells do not form junctions or exhibit complex architecture, and therefore cell division in this cell type occurs in the context of two dimensions. Similarly the analysis of cell division in Drosophila embryos has concentrated on stages of development prior to the establishment of septate junctions and permeability barriers. As mitosis and cytokinesis are organized at the level of the septate junctions (SJs) (Kojima et al., 2001; Gibson et al., 2006), and the vertebrate tight junctions (TJs) (Reinsch and Karsenti, 1994; Kojima et al., 2001), this raises the question of how cell division is targeted to these regions and adapted to accommodate junctions and cell architecture.

In both Drosophila and vertebrate epithelia, junction integrity is maintained throughout cell division (Baker and Garrod, 1993; Reinsch and Karsenti, 1994; Baye and Link, 2007). How existing junctions are maintained as new junctions are established is unknown. In cells where the permeability barrier is essential for tissue function, the SJs and TJs are likely maintained during cytokinesis for the survival of the cell and organism.

In Drosophila epithelial cells, the permeability barrier is formed by pleated septate junctions (SJs) (Tepass and Hartenstein, 1994; Tepass et al., 2001). They form in most polarized epithelia and are essential in some tissues, such as the salivary gland and glia, to prevent the flow of...
solute across the membrane. Ultrastructurally, SJs are characterized by electron dense material, or septae, that reside between adjacent cells and have the appearance of ladder rungs. The SJs wrap around the cell in strands that reside in a basolateral domain below the apical adherens junctions (Tepass and Hartenstein, 1994).

The SJ is generated by a core complex of proteins that include NeurexinIV (NrxIV), Coracle (Cor), Neuroglian (Nrg), Nervana 2 (Nrv2) and \(\alpha\)-ATPase (\(\alpha\)-ATP) (Genova and Fehon, 2003; Paul et al., 2003). Without these core proteins the septae do not form and the permeability barrier is lost, resulting in embryonic lethality or cell lethality in somatic clones (Lamb et al., 1998; Genova and Fehon, 2003; Paul et al., 2003). The SJ core complex is co-dependent for localization, as loss of any one of the core proteins leads to misplacement of the others (Lamb et al., 1998; Genova and Fehon, 2003; Paul et al., 2003).

Here, the columnar epithelia of the wing imaginal disc are used as an example of an architecturally complex cell to map cell division with respect to the presence of complex junctional domains. The columnar epithelia are an ideal system to assay for cell division as they undergo multiple rounds of cell division, and both have adherens and septate junctions.

This chapter maps the localization of known mitotic markers with respect to the septate and adherens junctions through all the phases of mitosis and demonstrates an association of cell division with the SJs. The orientation and progression of mitosis through to cytokinesis is coordinated with cell architecture (Eggert et al., 2006), and this work suggests that in architecturally complex cells the SJs play an important role in mitosis and cytokinesis.
**Materials and Methods**

**Fly Strains**

Stocks referred to as wildtype are w^{1118} or the GFP fusion protein lines outlined below. For wild type analysis of cell division, we used GFP fusion proteins from the Bloomington stock center or the Fly Trap Project, Sdc-GFP (CC00871), Dlg-GFP (CC01936), Zip-GFP (CC01626), NrxIV-GFP (CA06597) (Buszczak et al., 2007) and Jupiter-GFP (G00147), Nrg-GFP (G00305), (Morin et al., 2001).

**Immunofluorescence**

Immunolabeling of third instar imaginal wing discs was carried out as described previously (Schulte et al., 2006). All images were generated on a Deltavision Restoration microscope (Applied Precision). Data was collected using a 60X (1.4 NA) oil immersion lens using a CoolSnap HQ digital camera. Data from all wavelengths was collected for each 0.2 um optical section before the next section was collected. SoftWorx (Applied Precision) software was used for deconvolution of 8-10 iterations using a point spread function calculated with 0.2 um beads conjugated with Alexa Fluor 568, 488 and 647 (Molecular Probes) mounted in Vectashield. Images were then exported to Photoshop CS4 for generation of figures.

Primary antibodies used in study were: mouse anti-Dlg 4F3 at 1:300 (Parnas et al., 2001), rat anti-DE-cadherin at 1:50 (Oda et al., 1994), mouse anti-Coracle (9C and C615-16B cocktail) at 1:500 each (Fehon et al., 1994), mouse anti-Gliotactin 1F6.3 at 1:100 (Auld et al., 1995) or rabbit anti-Gliotactin at 1:300 (Venema et al., 2004), rabbit anti-Anillin 1:600 (Goldbach et al., 2010), mouse anti-γ-tubulin 1:600 (Abcam), mouse anti-acetylated tubulin 1:600 (Abcam), mouse anti-α-tubulin 1:600 (Sigma), rabbit anti-phospho-Histone 3 1:600 (Abcam). All the
following secondary antibodies were used at 1:200 dilution: goat anti-mouse, anti-rabbit or anti-rat conjugated to one of Alexa Fluor 488, Alexa Flour 568 and Alexa Fluor 647 or Cy5, (Molecular Probes). Nuclei were detected with DAPI at 1:1000.

**Assaying mitotic stages**

GFP line progeny raised at 25°C. Imaginal discs were isolated from wandering 3rd instar larvae and fixed following standard protocols (Schulte et al., 2006). Antibody stains were repeated a minimum of twice and 10 imaginal discs were imaged for each experiment to control for consistency. Discs that were damaged or otherwise varied from other wildtype samples were excluded.

For assaying the stage of mitosis the wing pouch was exclusively imaged. Images were collected using a 1024X1024 pixel area with a 60X objective (122X122 microns) across the dorsal/ventral border. Each stage of mitosis was determined as follows: metaphase and anaphase with DAPI, \( \gamma \)-tubulin, phospho-Histone3 immunofluorescence; telophase A-C with Dlg, \( \gamma \)-tubulin, DAPI immunofluorescence.
**Results**

**Mapping mitosis in the columnar epithelia in the imaginal wing**

To understand how mitosis proceeds in the columnar epithelia of the wing imaginal disc, a map of mitosis first needed to be developed to follow the spindle, midbody and nuclei. The goal was to develop a mapping system that could be used later to correlate the position of specific mitotic markers and stages with the septate and adherens junctions.

In the columnar epithelia, the nucleus is localized to the basal side of cell during interphase. At the onset of mitosis it transitions apically approximately 30 μm, the average height of the columnar epithelia, to the septate junction domain (Gibson et al., 2006).

Phosphorylated Histone 3 (PH3) was used as a primary staging tool for mitotic cells. Using PH3, cells were grouped into prophase (Figure 2.1A-B), metaphase (Figure 2.1C-D), anaphase (Figure 2.1E-F), or telophase (Figure 2.1G-L). Immunolabeling of α-tubulin was used to identify the spindle (Field et al., 2005), and a microtubule associating protein (Jupiter-GFP) was used to identify the midbody (Karpova et al., 2006). At prophase, the nucleus has transitioned to the apical side (Figure 2.1B, arrowheads) and the top of the cell has widened in diameter to accommodate the nucleus (Figure 2.1A). The increase in diameter is correlated to the absence of concentrated α-tubulin seen at the apical side of neighbouring interphase cells (Figure 2.1A, arrows). The concentration of α-tubulin found apically in an interphase cell is referred to as the “tubulin cap” and is absent in dividing cells (Figure 2.1A, arrows, α-tubulin). By metaphase the astral microtubules of the spindle are visible and are marked by Jupiter and α-tubulin (Figure 2.1C, arrows; Figure 2.1D, arrowheads). At anaphase, the central microtubules are more prominent, marked by Jupiter-GFP and α-tubulin, and the nuclei have separated (Figure 2.1E, arrows, Figure 2.1F, arrowheads).
Cells were further classified into three distinct stages of telophase; early telophase (telophase A, Figure 2.1G-H), mid telophase (telophase B, Figure 2.1I-J), and late telophase (telophase C, Figure 2.1K-L). This was necessary to describe and accurately portray the events that occur in these structurally complex cells during cytokinesis.

**Localization of the midbody in columnar epithelia**

During telophase A, (Figure 2.1G-H) both Jupiter and α-tubulin label the midbody (arrowhead) and the mitotic spindle (arrow) respectively. Jupiter highlights the spindle faintly and the midline furrow. By telophase B, α-tubulin forms two bridges on either side of the midbody that will persist until telophase C. This is referred to as the “midbody bridge” (Figure 2.1I-J, arrows). Jupiter at this stage marks the condensed midbody (Figure 2.1I-J, arrowheads). By telophase C, α-tubulin still marks the midbody bridge and Jupiter highlights the midbody, however, the chromosomes are no longer positive for PH3. The nuclei start to descend basally (Figure 2.1J, PH3) around telophase B, and this basal migration continues through telophase C. During the remainder of telophase C (Figure 2.1K-J) α-tubulin (arrows) and Jupiter (arrowheads) are highly concentrated at the top of cells, apical to the zonula adherens. Following cytokinesis, α-tubulin and Jupiter “reset” and are found apical the zonula adherens of the newly divided cells. They remain concentrated here until the next round of cell division (Figure 2.1A, arrows).

**Cell division is maintained at the septate junctions**

To further map and characterize the stages of cell division in the context of the SJs, imaginal wing discs expressing GFP tagged Neuroglian (Nrg-GFP) or NeurexinIV (NrxIV-GFP) were immunolabeled for PH3 and γ-tubulin.
At interphase the columnar epithelia have a vase like shape, with a small apical diameter (Figure 2.2A, arrowheads; Figure 2.2O, interphase). When the nucleus transitions apically to the SJ domain at the onset of mitosis (Figure 2.2A-B, arrows; Figure 2.2O), the cell diameter increases to accommodate the nucleus, and continues to increase its diameter through metaphase (Figure 2.2C, arrowheads), until it reaches its maximum diameter during anaphase (Figure 2.2E, arrowheads). During telophase A the ingression furrow is seen to form on either side of the cell, visible at the septate junctions as a small indentation (Figure 2.2G, arrowheads; Figure 2.2O, telophase A). The nuclei are still localized to the SJs at this point (Figure 2H, arrows; Figure 2.2O, telophase A). Through telophase A, the ingression furrow continues to contract (Figure 2.2I, arrowheads) and the nuclei start their basal migration (Figure 2.2J, arrows).

By telophase B, the SJs have formed a more structured furrow (Figure 2.2K, arrowheads; Figure 2.2O, telophase B) and the midbody bridge is visible (Figure 2.2K, asterisk). The nuclei continue to descend basally (Figure 2.2L, arrowhead) and no longer strongly express PH3. As the nuclei descend, the apical diameter of the two daughter cells decreases (Figure 2.2K compare to Figure 2.2I).

By telophase C, the nuclei are no longer visible at the SJ domain, and are no longer positive for PH3 (Figure 2.2M-N, PH3). The ingression furrow had formed a structured tunnel (Figure 2.2M, arrowheads; Figure 2.2O, telophase C), through which the midbody bridge is visible (Figure 2.2M-N, asterisk). The nuclei have descended below the SJs (Figure 2.2N, arrowheads; Figure 2.2O, telophase C), and the cell diameter at this level has returned to the interphase size (Figure 2.2M).
The descending cleavage furrow associates with the septate junctions

Cell division is localized to the SJ domain and the ingression furrow forms at the SJs (Figure 2.2) (Gibson et al., 2006). After a benchmark was established to identify all the mitotic stages of the cell cycle, a closer look was taken at cytokinesis. Cytokinesis itself consists of 4 distinct, temporally controlled phases: formation of the cleavage furrow, assembly of the contractile ring and ingestion, midbody formation and abscission (Glotzer, 2005; Eggert et al., 2006). Since the ingression furrow is associated with the SJs, it raised the question of whether the rest of cytokinesis could be mapped to the SJs as well.

To mark the initiation of the cleavage furrow, Syndecan endogenously tagged with GFP (Sdc-GFP) (Morin et al., 2001), was used. Syndecan is a transmembrane proteoglycan that has a role in synapse formation (Johnson et al., 2004; Steigemann et al., 2004; Rawson et al., 2005), and interacts in a complex with NrxIV (Banerjee et al., 2010). In columnar epithelia, Syndecan marks the initiation of the cleavage furrow that will descend basally, referred to as the descending cleavage furrow (DCF) (as opposed to the ascending cleavage furrow (ACF), discussed later) (Figure 2.3C,F, arrowheads; Figure 2.3G, red) and is found in conjunction with non-junctional Dlg (Figure 2.3C,F, arrow; Figure 2.3G, grey).

The descending cleavage furrow marker is first seen at the cleavage furrow during metaphase (Figure 2.3B-C, arrowheads), basal to the SJs but overlapping with non-junctional Dlg (Figure 2.3B, arrow). This appears to occur earlier than in architecturally simple cells, where the ingression and cleavage furrow are specified by the spindle during chromosome separation (Bringmann and Hyman, 2005; D'Avino et al., 2005; Zavortink et al., 2005; von Dassow et al., 2009). In metaphase cells, non-junctional Dlg is localized just below the nucleus to mark the site where the new bicellular membrane and SJs will be forming (Figure 2.3B-C). This pattern of
Sdc-GFP and Dlg continues through anaphase (Figure 2.3D-F) to telophase A (Figure 2.4A-D), where the descending cleavage furrow begins bisecting the cell (Figure 2.4C-D, arrowheads).

By telophase A, the descending cleavage furrow (Figure 2.4A-D, arrows) has reached the basal domain of the SJ (Figure 2.4A-D, arrowheads) and the cell has begun the second phase of cytokinesis, ingression. An enface view of the SJs shows that the membrane on either side of the furrow has begun to contract towards the center of the cell. At this focal plane, Dlg is marking the SJ and highlights this membrane contraction, which appears en face to be “pinching in” on either side (Figure 2.4A-B, arrowheads). The horizontal side projection of a metaphase cell illustrates how non-junctional Dlg overlaps with the initiation site of the descending cleavage furrow (Figure 2.3C). By telophase A, the descending cleavage furrow still overlaps with non-junctional Discs large and occupies the lower half of the septate junctions (Figure 2.4D, arrow).

By telophase B, an ascending cleavage furrow has formed apical to the midbody and expands towards the zonula adherens (Figure 2.4E-F, arrow). Both cleavage furrows now span the entire SJ domain (Figure 2.4G-H, arrows), except for a region around the midbody (Figure 2.4E, arrow). Non-junctional Dlg also appears more concentrated at the descending cleavage furrow, in the SJ domain (Figure 2.4G-H, arrows). The opposing arrangement of the cleavage and ingression furrows (Figure 2.4E, arrowheads; Figure 2.4H, arrows) will evolve into a structured tunnel around the midbody, referred to as the septate junction tunnel due to its localization within the septate junction domain. The formation of the septate junction tunnel, also known as the intercellular bridge (Poodry and Schneiderman, 1970; Haglund et al., 2010), corresponds to the third phase of cytokinesis, formation of the midbody.

By telophase C, as determined by reduced immunolabeling for PH3 and the structured SJ tunnel (Figure 2.4I, arrowhead), Sdc-GFP is reduced at the bicellular membrane directly below
the SJ tunnel (Figure 2.4J, arrows). A horizontal side projection of a telophase C cell shows that Sdc-GFP now occupies an arc at the apical domain of the SJs, in the same region of the midbody (Figure 2.4K, arrow; Figure 2.4L, arrow) and only overlaps with the SJs at the edges (Figure 2.4K, arrowheads).

A vertical side projection further illustrates the SJ tunnel that has formed around the midbody (Figure 2.4L, arrowheads) and suggests that the SJs are forming a three dimensional tunnel that is involved in maintaining contact between daughter cells and may be an important structural component during cytokinesis.

The centrosomes transition apically during telophase

After demonstrating that cell division occurs at the septate junctions, the next step was to investigate the localization of the machinery associated with cell division and cytokinesis. The first structure examined was the centrosome.

At the onset of prophase in wildtype columnar epithelia, the centrosomes, immunolabeled for γ-tubulin, are localized to the SJ domain but do not co-localize with the SJs. They remain in this region through to anaphase (data not shown) until telophase A, when the centrosomes begin to migrate apically (Figure 2.5A-B, arrows). As cells progress to telophase B, the centrosomes ascend to the apical side of the cell above the midbody and the septate junctions (Figure 2.5C, arrowheads; Figure 2.5E, arrows). By telophase C, the centrosomes are localized apical at either end of the midbody (Figure 2.5F, H, arrows). Note that this is the same level that γ-tubulin localizes to in adjacent interphase cells (Figure 2.5E, asterisks).

The localization of the centrosomes to the apical side of the cell during interphase was intriguing, especially as the centrosomes migrate to the SJ domain for the duration of cell
division and then return to their apical location. This is reminiscent of what is seen in vertebrate epithelial cells, where the centriole is commonly localized to the apical side of the cell where it can form the basal body for the primary cilia (Vorobjev and Chentsov Yu, 1982; Reinsch and Karsenti, 1994; Pan and Snell, 2007). Although Drosophila epithelia do not have primary cilia or centrioles (Basto et al., 2006), the localization of \( \gamma \)-tubulin to the apical side of interphase columnar epithelia suggests that an analogous structure may form. Imaginal discs were also immunolabeling for two centriole markers, SasB and Ana3 (Rodrigues-Martins et al., 2007; Stevens et al., 2009), to confirm the lack of centrioles in columnar epithelia (data not shown).

As the localization of \( \gamma \)-tubulin to the apical side of the cell in the absence of centrioles was intriguing, the centrosomes were characterized further.

**Polo kinase localizes to the apical side of the cell during interphase**

To confirm the observation of a centriole-like structure at the apical side of interphase cells, a second non-tubulin centrosome/centriole marker, Polo kinase, endogenously tagged with GFP (Polo-GFP) (Moutinho-Santos et al., 1999), was used. Polo like kinase (Plk) was originally characterized for mutations that caused abnormal mitosis phenotypes, including disorganized spindle poles and abnormal chromosome segregation (Sunkel and Glover, 1988; Llamazares et al., 1991). It has been shown to associate with multiple components of the mitotic apparatus, including the centrosomes, centromeres, spindle midzone, the midbody, kinetochores, and the central spindle (Logarinho and Sunkel, 1998; Moutinho-Santos et al., 1999; Archambault and Glover, 2009). The distribution of Polo kinase was characterized in dividing and interphase columnar epithelia in order to determine if it would mimic the localization pattern of \( \gamma \)-tubulin.
In interphase cells, Polo-GFP localizes above the zonula adherens in the same manner that was seen with γ-tubulin. (Figure 2.6B, arrows). In dividing columnar epithelia, Polo-GFP associates with the centrosomes and showed a similar localization pattern as γ-tubulin. It also localized to the spindle and the ingression furrow (Figure 2.6). At prophase and metaphase, Polo-GFP marks the plane of cell division (Figure 2.6A-D, arrowheads) which bisects the metaphase plate (Figure 2.6C-E, DAPI, arrowheads). Late in metaphase Polo-GFP associates with the spindle (Figure 2.6E-F, arrows) and centrosomes (Figure 2.6E-F, arrowheads). By early anaphase Polo-GFP clearly marks the spindle (Figure 2.6G-H, arrows) and centrosomes (Figure 2.6G-H, arrowheads). By mid-anaphase, the spindle microtubules have retracted so that the centrosomes and spindle almost overlap (Figure 2.6I-J, arrowheads). As the cells transition into telophase, Polo-GFP (Figure 2.6K-L, arrowheads) associates with the ingression furrow (Figure 2.6K, arrows). By telophase B, Polo-GFP (as staged by the proximity of the descending nuclei to the SJs) is still localized to the ingression furrow, and forms a tight ring, still within the SJ domain (Figure 2.6M-N, arrowheads).

The localization of Polo-GFP and γ-tubulin to the apical side of the cell during interphase further suggests that Drosophila epithelial cells have a centriole like structure.

The midbody and contractile ring localize to the septate junction tunnel during telophase

To further investigate what role the SJ tunnel could be playing in cytokinesis the localization of the contractile ring and midbody were next mapped in dividing columnar epithelia.

In order to determine where the contractile ring and midbody were localized with respect to the SJ tunnel, we looked at Anillin immunolabeling in conjunction with the SJ markers Dlg and Gliotactin. Anillin is a scaffolding protein that binds F-actin, Myosin II, and septins, and is a
major organizing and structural component of the contractile ring necessary for furrow stability (Field and Alberts, 1995; Oegema et al., 2000; Somma et al., 2002; Echard et al., 2004; Straight et al., 2005; Zhao and Fang, 2005; D'Avino et al., 2008; Almonacid et al., 2009). To mark the midbody, we used the microtubule associating protein Jupiter endogenously tagged with GFP (Karpova et al., 2006). At the start of telophase A, the contractile ring initially appears as two bands (Figure 2.7 A-B, arrows) on either side of the ingression furrow. A side projection shows that the Anillin bands project through the SJ domain (Figure 2.7C, arrows). This also corresponded to the localization pattern of Polo-GFP at the ingression furrow during telophase as was expected (Logarinho and Sunkel, 1998).

During telophase B (Figure 2.7D-F), the membrane has furrowed to form a more structured tunnel (Figure 2.7D, arrowheads). At this stage, Anillin does not localize to a single band throughout the SJ tunnel, but forms bands at either end (Figure 2.7D, H, arrows). The two bands of the contractile ring are localized within the tunnel and around the midbody as shown by Jupiter-GFP (Figure 2.7D, arrows). This indicates that the contractile ring in columnar epithelia cells has two ring components, not one as shown in S2 cells (Straight et al., 2005) and embryonic epithelia (Field et al., 2005). As the ingression furrow continues to contract through telophase C, Anillin can be seen in the four corners of the tunnel (Figure 2.7E, I, arrows). Gliotactin is found on either side of the ingression furrow at this stage and highlights the membrane as the two sides of the ingression furrow start to meet (Figure 2.7E, arrowheads). A side projection shows that Gliotactin (Figure 2.7F, arrowheads) does not overlap with the contractile ring (Figure 2.7F, arrows).
Not surprisingly these results show that the contractile ring is also localized to the SJ domain. A novel result obtained from this analysis is the more complex nature of the double contractile ring seen in columnar epithelia that is not present in dividing embryonic epithelia or S2 cells.

**Zipper localizes to the ingression furrow and further illustrates the complex nature of the contractile ring in columnar epithelia.**

The second contractile ring marker investigated was Zipper/myosin II heavy chain, using an endogenously tagged fusion protein (Zipper-GFP). Zipper is the heavy chain of non-muscle myosin II and is necessary for formation of the contractile ring and furrow ingression (Straight et al., 2005). The initial goal was to confirm Zipper showed the same localization pattern as Anillin through cytokinesis.

During interphase, Zipper is localized to the cell membrane at the adherens junctions (Figure 2.8A, asterisk) until the end of metaphase (Figure 2.8A, arrowheads). During anaphase, Zipper colocalizes with Anillin at the presumptive cleavage furrow (Figure 2.8B, arrowheads) and continues this colocalization through telophase A (Figure 2.8C, H, arrowheads).

By telophase B (Figure 2.8D, I, arrowheads), Zipper still localizes to the ingression furrow, but no longer colocalizes with Anillin, which has separated into two rings (Figure 2.8D, I, arrowheads). This separation is even more visible in telophase C (Figure 2.8E,J, arrowheads) where Zipper and Anillin are localized to discrete and distinct domains at the SJ tunnel (the ingression furrow and at the four corners of the SJ tunnel respectively). Zipper also persisted at the ingression furrow past Anillin into late telophase C (Figure 2.8F, arrowhead) and formed a ring structure at the site of abscission (Figure 2.8G, arrowheads), similar to the ring formed by Polo-GFP during telophase B (Figure 2.6M-N, arrowheads).
The difference in localization seen between Anillin and Zipper at the ingression furrow indicates that the contractile ring is targeted to the SJ domain, but also that the organization of the contractile ring is more complicated than illustrated in S2 cells.

The adherens junctions reform in late telophase

In the Drosophila embryo, the adherens junctions are dynamically reorganized during cell division. Both actin and Zipper, which normally localize to the adherens junctions during interphase, transition between the contractile ring and zonula adherens during cytokinesis (Tepass, 2002). In columnar epithelial the adherens junctions are located apical to the SJs in a tight domain of no more than 0.4 \( \mu \)m, and both Zipper and actin are localized to this region during interphase (Tepass, 2002) and metaphase (Figure 2.8A, zipper, arrowhead). Due to the interaction of actin and Zipper with the adherens junctions, dividing cells were assayed for an association between the contractile ring and the adherens junctions. Dividing cells were also assayed to determine when the adherens junctions reformed at the new bicellular membrane.

Columnar epithelia were immunolabeled for the adherens junction markers E-cadherin and phosphorylated Src (p-Src), as well as the SJ markers Gli and Nrg-GFP, and the contractile ring markers Anillin and Zipper. During telophase A, the contractile ring, as shown by Zipper and Anillin immunolabeling, is established apical to the septate junction domain (Figure 2.9F-G, arrowheads) and appears to associate with the adherens junctions (Figure 2.9F-G, arrows). By telophase C the new bicellular adherens junctions had begun to form (Figure 2.9C, E, arrowheads) apical to the contractile ring (Figure 2.9C-E, arrows). The ingression furrow is still present at the SJs but now p-Src labels the adherens junction forming above the SJ tunnel (Figure 2.9I, arrowheads). By late telophase C, the adherens junction (Figure 2.9J, pSrc) is visible above
the SJ tunnel, which is still present in the SJ domain (Figure 2.9J, arrowheads). This indicates that the new adherens junctions are established during telophase B, as the ascending cleavage furrow is forming.

Although the adherens junctions shows some association with the contractile ring, they are only slightly stretched in diameter (Figure 2.9A, arrowhead), when compared to the SJs (Figure 2.9B, G, Gli, Dlg) indicating that although the top of the assembling contractile ring overlaps with the zonula adherens, cell division in columnar epithelia is otherwise centered at the SJs.

**Cell division in peripodial cells**

The focus of this chapter is cell division in architecturally complex columnar epithelial cells that exhibit an extensive apical/basal junctional domain, but were the patterns and structures seen modifications to accommodate the junctional domains or height? Would a more squamous-like epithelium show the same pattern of cell division? The Drosophila wing imaginal disc consists of two tissue types; columnar epithelia and the over lying peripodial cells. Peripodial cells are polarized, squamous-like epithelial cells that have apical basal polarity and junctional integrity, but are flattened, occupying approximately 1 micron, and therefore do not undergo apical nuclear migration or transition of the centrosomes. As a comparison, the ingression furrow, contractile ring, and tubulin bridge were assayed in dividing peripodial cells. Dividing peripodial cells posses a SJ tunnel (Figure 2.10A-C, arrows) and tubulin bridge (Figure 2.10A-C, arrowheads) both formed during telophase. The contractile ring, immunolabeled using Anillin, also localized to the SJ tunnel in two bands, as seen in the columnar epithelia (Figure 2.10A-C, asterisk). The Anillin and Zipper contractile ring subdomains localized to the same regions in peripodial as was observed in columnar epithelia (Figure 2.10D), with Anillin localizing to the four corners of the
SJ tunnel (Figure 2.10D, arrow, asterisk) and Zipper to the ingression furrow (Figure 2.10D, arrowhead). Since cytokinesis in peripodial cells mimics the patterns seen in columnar epithelia without interkinetic nuclear migration, this suggests that the localization of the contractile ring and cell division to the SJ domain is a result of junctional architecture and not a result of the height of the columnar epithelia.
Discussion

The goal of this chapter was to look at cell division in architecturally complex cells that also exhibit a complex organization of junctions, and determine if and what modifications had been made in cell division and cytokinesis to accommodate those architectural complexities. In order to address how cell division proceeds in cells with extensive junctional domains, the understanding of mitosis and cytokinesis needed to be expanded beyond previous work in S2 embryonic epithelia. Columnar epithelia cells are both architecturally complex and possess a complex organization of junction. This chapter maps all of mitosis and cytokinesis in columnar epithelia, and demonstrates that the major structural components and benchmarks of cell division are associated with the SJ domain. Below, the major events in cell division and their relationship to the septate junction domain are discussed.

Localization to the septate junction domain

A consistent theme for cell division in architecturally complex epithelial cells is the apical migration of the nucleus at the onset of cell division (Morris, 2003; Whited et al., 2004; Xiang and Fischer, 2004; Starr and Fischer, 2005; Gibson et al., 2006; Wilhelmsen et al., 2006). In vertebrate neuroepithelial progenitor cells and MDCK cells, mitosis always occurs at the apical surface and replication occurs at the basal cell surface (Frade, 2002; Baye and Link, 2007). As demonstrated in Figure 2.1 and 2.2, and suggested in previous work (Gibson et al., 2006), this nuclear migration is also conserved in Drosophila columnar epithelia. The localization of cell division in Drosophila columnar epithelia and MDCK cells to the SJ and TJ domains respectively, also suggests there is a conserved recruitment of junctional domains for
establishing the vertical plane of cell division (Reinsch and Karsenti, 1994; Gibson et al., 2006; Baye and Link, 2007; Schenk et al., 2009).

Nuclear migration and positioning of the nucleus in both vertebrates and invertebrates occurs through the activity of the microtubule-based motor proteins Dynein and Kinesin (Morris, 2003; Whited et al., 2004; Xiang and Fischer, 2004; Starr and Fischer, 2005; Wilhelmsen et al., 2006). The literature suggests that Dynein is responsible for the apical movement (towards minus end) of the nuclei and Kinesin is responsible for the basal (towards positive end) (Fan and Ready, 1997; Whited et al., 2004; Baye and Link, 2008). It remains to be seen whether Dynein-Dynactin and Kinesin are involved in nuclear migration in the wing imaginal disc.

As well as migration of the nucleus, the movement of the centrosome markers γ-tubulin and Polo kinase from the apical side of the cell to the SJs was observed during cell division. At the onset of mitosis, the centrosomes migrate from above the zonula adherens to the SJs and during telophase each centrosome migrates back. This migration pattern parallels the apically localized centrioles during mitosis in vertebrate epithelial cells (Reinsch and Karsenti, 1994; Baye and Link, 2007; Schenk et al., 2009). It has been suggested that in vertebrate cells, nuclear migration occurs so that the nucleus can meet up with the apical centrioles (Reinsch and Karsenti, 1994).

One key difference between the migration of the centrosome markers in Drosophila and the centrioles in vertebrates is that in vertebrate epithelial cells, the migration of the centrosome at the end of cytokinesis corresponds to the repositioning of the centrioles, and Drosophila epithelial cells do not posses’ centrioles (Basto et al., 2006). However, the presence of centrosome markers that follow the same migration and localization pattern of vertebrate epithelial, and the presence of a centriole-like microtubule organizing centre (MTOC) in tracheal
cells (Brody et al., 2010), suggests Drosophila columnar epithelial cells posses a centriole–like structure.

The migration of the nuclei and centrosomes in Drosophila epithelia supports the idea that the localization of cell division to the correct vertical domain is an important cell division theme in architecturally complex cells.

**Establishing the cleavage furrow**

Once the plane of cell division is established at the septate junction domain, cells undergo mitosis and enter cytokinesis, which involves formation of the cleavage furrow, contractile ring and ingression furrow, in that order (Glotzer, 2003). The localization of these steps to the SJ domain indicates the SJs may be acting as a scaffold for cell division.

How do cells organize the plane of cell division and target it to the SJ domain? Coordination of the spindle microtubules with the cell cortex are an essential component. The position of the central spindle and astral spindle microtubules act as cortical cues that specify the cleavage furrow and target other cortical cues, such as Rho A, which signal for assembly of the contractile ring. However the mechanism by which the spindle is targeted is unknown (Betschinger and Knoblich, 2004). It is also unknown how the spindle might be targeted to the SJ domain in columnar epithelia, however, one potential link between the SJs and organization of the plane of cell division is the SJ associated protein Dlg. During the generation of sensory organ precursors through asymmetric cell division in the imaginal wing disc, Dlg organizes the plane of cell division (Bellaiche et al., 2001; Albertson and Doe, 2003; Siegrist and Doe, 2005). It is possible that Dlg is also responsible for establishing the plane of cell division during symmetric cell division in the columnar epithelia. Lethal giant larvae (Lgl) is another SJ associated protein that
interacts with Dlg in a complex that is responsible for establishing apical/basal polarity (Bilder et al., 2000), and is necessary for establishing the asymmetric plane of cell division in embryonic neuroepithelia (Lu et al., 2001; Petronczki and Knoblich, 2001). Lgl is there for another potential link between the SJs and organizing the plane of cell division. The potential for both Lgl and Dlg as mediators of cell division in columnar epithelia is discussed at greater length in Chapters 4 and 5.

The assembly of the contractile ring goes hand in hand with the specification of the cleavage furrow (D'Avino et al., 2005). The recruitment of cleavage furrow factors and activation of RhoA GTPase at the equatorial cortex leads to assembly of the contractile ring through the recruitment of Zipper, Anillin and actin (D'Avino et al., 2005). This recruitment somehow has to be linked to the SJ domain. Once again Lgl is a potential link, due to its localization to the SJ domain in polarized epithelia, and its role in restricting Zipper during asymmetric cell division in neuroepithelia (Strand et al., 1994; Lu et al., 2001; Petronczki and Knoblich, 2001).

A novel observation obtained from this analysis is the more complex nature of the double contractile ring seen in columnar epithelia that has not been observed in dividing embryonic epithelia or S2 cells. The separation of Zipper and Anillin into sub-compartments (Figure 8E) of the contractile ring further illustrates the added level of complexity in columnar epithelia. Each ring of the Anillin seen at telophase B corresponds to the point of the SJ tunnel that contacts each daughter cell, suggesting the ring exists in two parts. Not only are there two rings, but Zipper is localized to the ingestion furrow and does not overlap with Anillin past telophase A. Anillin acts as a link between actin, non-muscle myosin II and the septin complex (Field and Alberts, 1995; Kinoshita et al., 2002; Straight et al., 2005) but their localization in late telophase likely
reflects the fact that Anillin has an early role in properly organizing the contractile ring and a later function in restricting Zipper contraction to the ingression furrow (Straight et al., 2005). The organization of Anillin to two rings that flank Zipper in telophase B-C in columnar epithelia would suggest that the antagonistic relationship between Zipper and Anillin is carried over into architecturally complex cells, where Anillin inhibits Zipper and restricts it to the ingression furrow.

Regardless, the division of the contractile ring seen in columnar epithelial allows for a more detailed analysis than is possible in S2 cells.

The descending and ascending cleavage furrows

The asymmetry of architecturally complex cells, and the fact that they maintain their junctions through cell division, means that new membrane synthesized at the cleavage furrow must take both the existing junctions and apical/basal polarity into account. The cleavage furrow can be subdivided into two sections the descending cleavage furrow and the ascending cleavage furrow. Both are established in the SJ domain, but the descending cleavage furrow assembles in a basal direction and the ascending cleavage furrow assembles apically towards the adherens junctions.

Non-junctional Dlg localizes to the initiation site of the descending cleavage furrow region at metaphase as shown in Figure 2.3. This association precedes the localization of septate junction core components NrxIV and Nrg. That Dlg is localized to the early descending cleavage furrow is that it may be necessary for establishing apical/basal polarity in the newly forming bicellular membrane, as Dlg is necessary for establishing apical/basal polarity during embryogenesis (Noirot-Timothée and Noirot, 1980; Woods and Bryant, 1991; Woods et al., 1996).
It should also be noted that the descending cleavage and ascending cleavage furrows coincide with the formation of the new membrane. The assembly of the new membrane is essential for the completion of cytokinesis and requires both the targeting of vesicle and their fusion to the new bicellular membrane. Although vesicle targeting and fusion are typically associated with abscission in less architecturally complex cells (Eggert et al., 2006), in the case of columnar epithelia, this coincides with the formation of the descending cleavage furrow early in mitosis instead. Syndecan is a Drosophila transmembrane proteoglycan necessary for axon guidance during embryogenesis (Rawson et al., 2005) that was found localized to the early descending cleavage furrow. Syndecan interacts with NrxIV during axon guidance (Banerjee et al., 2010), so it is possible that Syndecan is associating with the SJs at the descending cleavage furrow.

One possible role for Syndecan at the cleavage furrow is the targeting and fusion of vesicles to newly forming membrane. In other cell types, the exocyst complex is responsible for targeting new membrane vesicles to the cleavage furrow and the SNARE complex is necessary for the fusion. However, it is not understood how the two components interact (Gromley et al., 2005; Eggert et al., 2006). Syndecans in general act as multifunctional co-receptors and ligands (Carey, 1997) and in Drosophila Syndecan acts as a ligand for the LAR receptor tyrosine phosphatases (Fox and Zinn, 2005). It is possible that Syndecan is mediating the targeting of the exocyst to the SNARE complex, however, further study is needed to determine whether Syndecan performs a functional role in cytokinesis, or whether its localization to the descending cleavage furrow is coincidental.
The midbody and intercellular bridge

In S2 cells, the contractile ring and midbody occupy the same region and are often referred to interchangeably (Straight and Field, 2000). The distinct localization of the contractile ring, midbody and midbody bridge seen in columnar epithelial is likely a direct effect of the more complex architecture compared to the overlapping seen in S2 cells (Straight and Field, 2000; Straight et al., 2005). The tubulin bridge reported here in Figure 2 supports this hypothesis, as it likely has a counter part in S2 cells condensed into the midbody-contractile, as the midbody is partly composed of tubulins and microtubules (D'Avino et al., 2005; Eggert et al., 2006)

However, the architecture of columnar epithelia allows this type of structure to be seen as a discreet component.

The SJ tunnel formed during cytokinesis likely corresponds to a structure referred to as the intercellular bridge, a structure that connects daughter cells during cytokinesis described through TEM studies in imaginal discs (Poodry and Schneiderman, 1970; Haglund et al., 2010). Whether these intercellular bridges are permanent structures or absolved during cytokinesis is still debated (Haglund et al., 2010), but the transient localization of the midbody marker Jupiter-GFP reported here supports the idea that the intercellular bridges are resolved during cytokinesis through abscission.

Abscission

The rings formed late in cytokinesis highlighted by Zipper-GFP and Polo-GFP suggest that the membrane in the SJ tunnel is being excised into one of the daughter cells. Figure 8H-K represents an excision model where the ring structure is due to the mechanism of abscision employed in columnar epithelia. The circularization of Zipper represents the two sides of the
ingression furrow fusing so it can be excised away from the newly formed bilayer. This ring is then is excised and possibly retained by one of the two daughter cells. The fusing of the ingression furrow into a circulized structure provides an explanation of how the cell can resolve the two opposing membranes of the ingression furrow without disrupting the new bilayer and junctions.

The adherens junctions and cell division
In embryonic epithelia and neuroepithelia, the adherens junctions play a critical role in organizing the plane of cell division. In neuroepithelia, the adherens junctions are necessary for maintaining symmetric cell division, and their absence is necessary for asymmetric cell division to proceed (Lu et al., 2001). In blastoderm epithelia, the adherens junctions are dynamically reorganized and cytokinesis is tied to the redistribution of Zipper and actin back to the zonula adherens (Tepass, 2002). By telophase C in columnar epithelia, the adherens junctions have formed (Figure 9E), whereas the septate junction tunnel is still associated with the contractile ring and midbody. Although we noted that cytokinesis is not localized to the adherens junctions, they still may have a role in the redistribution of the contractile ring components Zipper and actin, as both are localized to the zonula adherens during interphase (Figure 9F) (Harris and Tepass, 2010). It is also possible that in the imaginal wing disc the adherens junctions are involved in the anchoring of the ascending cleavage furrow or the organization of other apically associated structures. It remains to be seen whether disruption of the adherens junctions will disrupt cell division in the columnar epithelia.
Do tight junctions perform a similar role in vertebrate epithelial cells?

A question that arises from this study is whether cytokinesis in vertebrate epithelia is localized to the tight junctions. Reinsch and Karsenti implicate that cell division was associated with the tight junctions as they show the ingression furrow forming at the tight junctions in Figure 9K in Reinsch and Karsenti, 1994. These observations suggest that cell division and the ingression furrow occur at the tight junctions. The extent of the functional roles that the tight junctions and their Drosophila equivalent, the septate junctions, have in mitosis and cytokinesis remains to be seen. Future analysis in this area will help determine whether the tight junctions perform a structural role in mitosis and in cytokinesis in particular.

Conclusion

In architecturally complex epithelia many aspects of cell division are modified to accommodate the presence of extensive junctional domains. This includes localization of the spindle, centrosomes, contractile ring, ingression furrow, and midbody to the SJ domain and provides insight into the relationship between the junctions and cell division. This chapter presents a map of mitosis and cytokinesis in wildtype columnar epithelia to be used as a comparison against mutant phenotypes seen in Gliotactin and NrxIV RNAi expressing discs.
**Figures**

**Figure 2.1 Localization of cell division markers in polarized epithelia**

The columnar epithelia of 3rd instar imaginal wing discs are shown at each stage of mitosis enface (left panels: A,C,G,I,J,K) and as a side projection (right panels: B,D,F,H,J,L). Cells were labeled for the microtubules, α-tubulin (α-tub, green), a midbody marker, Jupiter-GFP (Jup-GFP, blue), and phosphorylated Histone 3 (PH3, red). En face views are a single 0.2 μm Z slice at the level of the mitotic spindle. Side projections represent the top half of the epithelia (~10-15 μm).

A-B) At prophase, the nucleus (PH3) transitions to the apical side of the cell as indicated by arrowheads in B and the presence of the α-tubulin “caps” in the surrounding interphase cells (A, arrows).

C-F) At metaphase and anaphase, α-tubulin and Jupiter-GFP mark the spindle (C,E, arrows). The spindle is localized to the apical domain (D,F, arrowheads).

G-H) At telophase A, α-tubulin and Jupiter still mark the remainder of the spindle and astral-microtubules (arrows). Jupiter also marks the cleavage furrow (G, arrowhead). Cell division is still localized to the apical domain (H, arrowhead).

I-J) By telophase B, α-tubulin marks the “midbody bridge” (I,J, arrows), which form on either side of the midbody marked by Jupiter-GFP (I,J, arrowhead). The nuclei have started to descend back to the basal side of the cell and no longer stain strongly for PH3 (J, asterisk).

K-L) At telophase C, the midbody (Jupiter-GFP, arrowhead) and midbody bridge (α-tubulin, arrows) are still visible. The nuclei are no longer positive for PH3, and continue to descend basally (L, asterisk).
M) A cartoon depiction of cell division in columnar epithelia. Mitotic stage is indicated below. The enface view is shown above each corresponding side view. Nuclei (blue), septate junctions (red), microtubules (black), centrosomes (orange), Gliotactin (green), midbody (charcoal grey).

At interphase (rest), the nucleus resides in the basal compartment of the columnar epithelia; the centrosome marker $\gamma$-tubulin (orange circle) is concentrated at the top of the cell along with a microtubule cap (black star) at the apical side of the cell. During prophase, the nucleus transitions to the SJ domain at the apical side of the cell and the membrane widens to accommodate. The centrosomes align to the cell cortex in the SJ domain and the microtubule cap disappears. By metaphase, the astral microtubules are visible and the chromosomes have aligned. The chromosomes separate during anaphase and the central spindle microtubules become visible. By telophase A, the cleavage furrow has formed and begins to ingress (enface), and the midbody assembles (charcoal grey). Between telophase B and C, the centrosomes migrate to the apical side of the cell, and microtubules form on either side of the midbody, potentially reforming the microtubules cap in each daughter cell (telophase B-C, black triangles). The dashed black line with arrow indicates the descending cleavage furrow forming basally between telophase A and C, and the solid black line with arrow above the midbody in telophase B with arrow indicates the ascending cleavage furrow.
Figure 2.2 The septate junctions and cell division

The columnar epithelia of 3\textsuperscript{rd} instar imaginal wing discs are shown at each stage of mitosis.

Cells were labeled for septate junction using the endogenously tagged markers Neuroglian-GFP (Nrg-GFP, red) or Neurexin IV-GFP (NrxIV-GFP, red), plus immunolabeling with antibodies to microtubules with \(\alpha\)-tubulin (\(\alpha\)-tub, green) and phosphorylated Histone 3 (PH3, blue) to characterize each stage of mitosis with respect to the septate junctions. En face views are a single 0.2 \(\mu\)m Z slice at the SJ domain. Side projections represent the top half of the epithelia (~10-15 \(\mu\)m).

A-D) During prophase and metaphase, cells increase their diameter at the SJ domain compared to neighboring interphase cells (A-D, arrowheads). The nucleus has migrated apically to the SJ domain (A-D, arrows). The metaphase plate is also visible (C, arrow).

E-F) During anaphase, the cell widens at the SJ domain even more (E,F, arrowhead) to accommodate the separating chromosomes (E,F, arrow).

G-H) At the onset of telophase A, the opposing sides begin to invaginate to form the ingression furrow (G, arrowhead). The nuclei are separated and the cell has reached its maximum diameter. The daughter nuclei are still localized to the SJs (G,H, arrows).

I-J) Late in telophase A, the ingression furrow has almost formed a tunnel (I, arrowhead). The nuclei have started to descend, and are located in the lower region of the SJ domain (J, arrows)

K-L) By telophase B, the ingression furrow has formed a tunnel (K, arrowhead). The midbody bridge is located within the tunnel (K, asterisk). The nuclei have descended further (L, arrow). PH3 is weaker by this stage (K,L, arrows).

M-N) By telophase C, the diameter of the cell at the SJs has decreased. The midbody bridge can still be seen within the tunnel (M, arrowheads). The nuclei have descended and are no longer
positive for PH3 (N, arrowheads). The midbody bridge remains with the SJ domain at the apical side of the cell (M, N, asterisk).

O) A cartoon depiction of cell division in columnar epithelia. The enface view is shown above the corresponding side view. Nuclei (blue), septate junctions (red), microtubules (black), centrosomes (orange), Gliotactin (green), midbody (charcoal grey). See details Figure 2.1, M.
Figure 2.3 Initiation of the descending cleavage furrow at metaphase and anaphase

Cells were imaged at anaphase and metaphase at the level of the SJs (z=57) and basal to the SJs where the descending cleavage furrow is forming (z=36). Each enface image represents a single Z slice of 0.2 microns. Side projections were generated from the regions marked by the dashed lines and shown below the related enface panels. The SJs were labeled using Discs-large immunolabeling (Dlg, green). The descending cleavage furrow was labeled using Syndecan endogenously tagged with GFP (Sdc-GFP, red) and phospho-Histone3 (PH3) labeled mitotic DNA.

A-C) During metaphase, the metaphase plate (arrowhead) is found at the level of the SJ domain (A, z=53). The prospective site of the descending cleavage furrow labeled with Sdc-GFP (arrowhead) is seen basal to the SJs (B, z=36). C shows the side projection of the metaphase cell with Sdc-GFP (arrowhead) forming a crescent shaped region under the nucleus. Non-junctional Dlg (B,C, arrows) is also found in this region.

D-F) In anaphase, Sdc-GFP (E, arrowhead) is still seen basal to the SJs and has not spread into the SJs (F, arrowhead). The separated chromosomes are still centered at the SJ domain (D, arrowhead). Non-junctional Dlg (F, arrows) is now more concentrated at the descending cleavage furrow.

G) Cartoon representations of the cleavage furrow at metaphase and anaphase. Sdc-GFP (red), the SJs (green), Gliotactin (yellow), nuclei (blue), non-junctional Dlg (grey), α–tubulin spindle (black), adherens junctions (purple). The dashed red lines indicate the cleavage plane where Sdc-GFP is accumulating.
Figure 2.4 Progression of the cleavage furrow during telophase

Cells were imaged at telophase A, telophase B, and telophase C at the level of the SJs (z=57 A,E,I) and the basal most part of the descending cleavage furrow (z=36, B,F,J). Each enface images represents a single Z slice of 0.2 μm. Side projections were generated for individual cells at each stage on two axis marked with dashed lines; one directly through the forming cleavage furrow (C,G,K) and one through the length of the dividing cell (D,H,L). Discslarge was used to mark the SJs and membrane, Syndecan-GFP (Sdc-GFP) marks the top of the descending cleavage furrow and phospho-Histone3 (PH3) marks mitotic DNA.

A-D) At telophase A, the Sdc-GFP labeled descending cleavage furrow has localized to the level of the SJ (A-D, arrows). The enface view at the SJs shows the initiation of the ingression furrow (A, arrowhead). Non-junctional Dlg also labels the descending cleavage furrow (B,D, arrowheads).

E-H) telophase B: At the apical point of the descending cleavage furrow and at the level of the SJs, the ingression furrow has formed a tunnel outlined by the SJs (E,G-H, arrowheads). Sdc-GFP is localized to the tunnel (E, arrow) but does not overlap with the ingression furrow on either side. Dlg and Sdc-GFP now have extensive overlap (F-H, arrows).

I-L) telophase C: The remnants of Sdc-GFP now resides in the SJ tunnel formed by the invading ingression furrow (I, arrows) and the ingression furrow has almost fully invaded the SJ tunnel (I, arrowhead). Sdc-GFP is flanked apically and basally by the SJs (K-L, Dlg, arrowheads). At this stage the nuclei are no longer positive for PH3.

Scale bars represent 5 microns unless otherwise indicated.
Cartoons: To the left of each panel is a cartoon representation of each stage. Sdc-GFP (red), SJ Dlg (green), nuclei (blue), non-junctional Dlg (grey), Gliotactin (yellow), $\alpha$-tubulin (spindle and alpha tubulin cap) (black), centrosomes (beige), adherens junctions (purple).
Cells from 3\textsuperscript{rd} instar imaginal wing discs were imaged at the level of the SJs and stained for the centrosomes ($\gamma$-tubulin), the septate junctions (Dlg-GFP, Nrg-GFP and Gliotactin), the contractile ring (Anillin) and the midbody (Jupiter-GFP). Between telophase A-C the centrosomes transition to the apical side of the two daughter cells. A-B represent telophase A, B-C shows late telophase B, and E-F shows telophase C.

A-B) A telophase A cell. A) The ingression furrow (arrowheads) is used to stage cells. $\gamma$-tubulin panel is taken at z67, slightly basal to the ingression furrow, shown at a focal plane apical most range of the SJs (z53). B) A side projection of panel A. During telophase A, the centrosomes are still in the SJ domain. One centrosome has begun to migrate apically (arrow). Arrowheads indicate position of the ingression furrow.

C-E) late telophase B cell. C) By late telophase B, the centrosomes have migrated above the midbody and are almost positioned directly above either end of the midbody (arrowheads). D) The ingression furrow has also condensed (arrow) around the midbody (arrowhead) immunolabeled for Jupiter. E) A side projection of the cell in C. The centrosomes have transitioned above the SJ domain (E, arrows) and are almost positioned above the midbody (Jupiter-GFP). The ingression furrow is indicated by Gliotactin (E, arrowhead). $\gamma$-tubulin is found concentrated at the apical side of interphase cells (asterisks).

F-H) A telophase C cell. F) By telophase C the centrosomes have migrated to either apical side of the midbody (F, arrows) G) The ingression furrow has condensed further (G, arrowheads) around the midbody (G, arrow, Jupiter). The contractile ring is less prominent (G, arrowhead, Anillin) when compared to telophase B. H) A side projection of the cell in F. The centrosomes (arrows) are positioned at the apical side of the cell directly above the midbody (H, Jupiter-GFP).
The centrosomes are almost level with the apical $\gamma$-tubulin shown in adjacent cells. Gliotactin marks the ingression furrow (H, Gli).
Figure 2.6 Polo-kinase associates with key structures throughout mitosis in columnar epithelial cells.

Cells from 3rd instar imaginal wing discs were imaged at the level of the SJs (Dlg, red) and assayed for Polo kinase (Polo-GFP, green) and DAPI (blue). Cells are shown from prophase through to telophase C. Enface panels are single Z slices. The white line indicates the axis of each corresponding side projection. Each side projections shows the top 3/4ths of the membrane.

A,B) prophase. A) Polo-GFP (arrowheads) marks the emerging spindle axis. B) Note the localization of Polo-GFP to the apical side of the cell in interphase cells.

C,D) Early metaphase. C) Polo kinase clearly marks the emerging spindle axis as a straight line (arrowheads). D) Note the straight line formed by Polo spanning the entire SJ domain (arrowheads).

E,F) Late metaphase. E) Polo kinase now marks the centrosomes (arrowheads) and the points where the spindle attaches to the chromosomes (arrows). F) In profile the centrosomes can be seen at the level of the SJs (arrowheads) and the microtubule projections indicating the forming spindle can be seen to overlap with the chromosomes (arrows).

G,H) Early anaphase. The chromosomes begin separating. Polo kinase marks the centrosomes (arrowheads) and the separating chromosomes (arrows).

I, J) Late anaphase. I) The spindle has contracted to the edge of the cell and now contact the centrosomes. A “triangle” is formed where the two meet (arrowheads). This is also illustrated in side projection (I, arrowheads).

K,L) telophase A. K) As the ingression furrow forms (Dlg, arrows) Polo kinase marks the position where the contractile ring will form (arrowheads). L) The side projection shows that this site still localizes to the SJ domain (arrowheads).
M,N)Late telophase B as determined by the position of the nuclei at the basal level of the SJ domain. The ingression furrow forms the SJ tunnel (Dlg, arrowhead). By this stage Polo kinase is still associated with the ingression furrow but has formed a ring (arrowhead, Polo-GFP). N) The Polo kinase ring is still localized to the SJs (arrowheads).
**Figure 2.7 The contractile ring localizes to the septate junctions during cytokinesis**

Cells from 3\textsuperscript{rd} instar imaginal wing discs were imaged at the level of the SJs (Dlg, Gliotactin) and assayed for contractile ring marker Anillin, the midbody marker Jupiter and septate junction markers Dlg and Gli.

Each enface image represents 0.2 micron z slice. Side projections show the first SJ domain, approximately 15 microns or the top 1/3 of the cell.

A-C) telophase A.  A) During telophase A, the ingression furrow forms at the level of the SJ (Dlg, arrow). Anillin (red, arrow) associates with the tip of the ingression furrow marking the assembly of the contractile ring.  B) The tip of the ingression furrow labeled with Anillin (red, arrow) forms at the bicellular SJ junction between the TCJ marked by Gliotactin (green, arrowhead). C) A side projection of B shows the contractile ring labeled with Anillin (red) span the entire septate junction domain (arrows), immunolabeled with Gliotactin (green).

D) telophase B. By late telophase B, the ingression furrow has formed a structured tunnel (arrowhead) labeled with the SJ marker, Dlg (green). The contractile ring labeled by Anillin (red) spans either end of the tunnel (arrow).

E-F) telophase C.  E) A telophase C cell immunolabeled with Anillin (red) and Gliotactin (green). Gliotactin marks the SJ tunnel at this stage and can be seen on either side of the ingression furrow (arrow). F) A side projection of E with E-cadherin (blue) showing the constriction of the contractile ring in relation to the adherens junctions and the SJ domain (Gli, arrowhead).

G,H,I) Cartoon depictions of telophase A, early and late telophase C respectively. Anillin (red), Gliotactin (green), midbody (grey), the membrane (black) and the nuclei (blue) are shown.
Figure 2.8 Zipper has a distinct localization pattern in columnar epithelial cells

Cells from 3\textsuperscript{rd} instar imaginal wing discs were assayed for the contractile ring markers, Zipper using an endogenous GFP tag (Zipper-GFP, green) and Anillin (blue) with immunolabeling at the level of the SJs with antibodies to Dlg (red). En face panels represent 0.2 micron z-slices.

A) metaphase. Zipper localizes apically (z18) at the level of the adherens junctions and is concentrated around the cell (arrowheads). Note the apical localization of Zipper in surrounding interphase cells shown at the adherens junctions (A, Zipper GFP, star)

B) anaphase. Zipper (arrowheads) is now localized to the SJs where the ingression furrow will be localized (arrows).

C) Late telophase A. Zipper and Anillin co-localize to the ingression furrow (arrowheads).

D) telophase B. Zipper and Anillin still localize to the ingression furrow (arrowheads). Anillin has started to concentrate into 4 corners of the septate junction tunnel.

E) telophase C. Zipper and Anillin still over lap at the ingression furrow (arrowheads) but also concentrate to distinct regions. Anillin is concentrated into four sections at the corners of the SJ tunnel and Zipper is associated with the ingression furrow.

F) Late telophase C. Anillin is almost absent at the ingression furrow (Anillin, arrowhead).

G) Abscission. Zipper is found in a ring at the remainder of the SJ tunnel (arrowheads). Anillin is undetectable.

H-K) Cartoon depiction of telophase A (H), telophase B (I), telophase C (J), Abscission (K). septate junctions (red), Gliotactin (dark green), Zipper (purple), Nuclei (dark blue), Anillin (light blue). See description in text.
**Figure 2.9 The adherens junctions form during telophase C**

Cells from 3\textsuperscript{rd} instar imaginal wing discs were imaged at the level of the adherens junctions and septate junctions and assayed for contractile ring markers Anillin and Zipper, SJ markers Dlg, Nrg, and Gli, and adherens junction markers E-cadherin and activated Src. Each image represents one Z slice.

A-B) The telophase A cell from Figure 7A, shown at the level of the SJs (z22) and the level of the adherens junctions (z13). Note that the adherens junctions occupy a smaller diameter in the dividing cell (arrowheads). The forming contractile ring is shown (arrows).

C-E) A telophase C cell shown at the level of the SJs (z22) and the level of the adherens junctions (z26). C) The new adherens junctions have started to form (arrowheads). The top of the contractile ring (arrows) breaches the adherens junctions. D shows the same cell at the level of the SJs, and E is a magnification of C.

F-G) A telophase A cell imaged at the SJs (Dlg) and immunolabeled for the contractile ring markers Zipper and Anillin. F) Note the apical localization of Zipper at the adherens junctions (arrows) also marking the top of the contractile ring breaching the adherens junctions domain.

G) At the level of the SJs the ingression furrow is shown by Dlg (arrowheads) and the contractile ring is visible (arrows)

H-J) A telophase A cell (H), an early telophase C cell (I) and a late telophase C cell (J). Arrowheads indicate the site of the new bicellular junction at the level of the adherens junctions (Src) and the ingression furrow at the SJs (Nrg-GFP). Note that the new adherens junctions form apically to the SJ tunnel (J, arrowheads).
Figure 2.10 Squamous peripodial epithelial cells show same division of contractile rings in telophase as the columnar epithelial cells

Peripodial cells from 3rd instar imaginal wing discs were imaged for the SJ proteins (Cora, Dlg; green), the contractile ring proteins Anillin (blue) or Zipper-GFP (red), and the midbody tubulin bridge (acetylated tubulin, red).

A) A dividing peripodial cell during telophase C. The midbody tubulin bridge (arrowheads) and contractile ring (star) are localized to the SJ tunnel (arrows).

B) A close up view of the SJ tunnel shown in A. Note the localization of Anillin (star) to either side of the tunnel (arrow).

C) A side projection of the tunnel shown in B. The tunnel is still associated with the SJs (arrow) in this squamous epithelia.

D) A telophase 3 peripodial cell stained immunolabeled with Zipper-GFP (red), Dlg (green) and Anillin (blue). Anillin associates with the four corners (star) of the SJ tunnel (arrow) and Zipper associates with the ingression furrow (arrowhead).
III. Gliotactin is essential to maintain the plane of cell division in polarized epithelial cell of the imaginal wing disc

Introduction

Architecturally complex cells undergo a series of coordinated cellular events during cell division, including nuclear and centrosomes migration, extensive remodeling of the membrane, and formation of a new bicellular membrane. In Drosophila columnar epithelia, nuclei transition from the basal side of the cell to the apical SJs at the onset of mitosis, so that the plane of cell division occurs exclusively within the SJ domain (Gibson et al., 2006). Although the microtubules and microtubule motor proteins Dynein and Kinesin, are implicated in nuclear translocation (Blagden and Glover, 2003; Whited et al., 2004; Fridolfsson and Starr, 2010), how the nucleus transitions apically and is held within the SJ domain is unknown.

In Drosophila epithelia, during late embryogenesis the SJs form a “ladder rung” like array of electron dense material between opposing bicellular membrane that acts as a permeability barrier between adjacent cells (Tepass and Hartenstein, 1994; Tepass et al., 2001). Components of the SJs in Drosophila that have been uncovered by mutational analysis include a core complex of proteins, Neurexin IV (NrxIV), Coracle (Cor), Neuroglian (Nrg) and the Na+/K+ ATPase pump (ATPα and Nervana 2), that are dependent on localization of one another to the SJ domain (Genova and Fehon, 2003; Paul et al., 2003). Another protein necessary for the maturation of the SJs and permeability barrier is Gliotactin (Gli), a SJ associated protein concentrated at the TCJ (Schulte et al., 2003). The TCJ forms at the convergence of three SJ domains, which turn to descend basally at the cell corners. It consists of electron dense material reminiscent of “plugs” that are thought to compress and hold the SJ strands in place (Schulte et al., 2003; Schulte et al., 2006).
Besides being necessary for permeability barriers, the SJs have been implicated in maintaining epithelial polarity in the embryo (Laprise et al., 2009), cardiac integrity (Yi et al., 2008) and regulating tracheal tube size (Paul et al., 2003; Llimargas et al., 2004). In addition, mutations in any one of the SJ proteins in the wing imaginal disc result in cell lethality. Since there is precedence for SJs to function outside the permeability barrier, this thesis addressed whether the SJ and TCJ had a functional role in maintaining the plane of cell division at the SJ domain. To determine if there was an effect on the plane of cell division, RNAi for the core SJ proteins and Gli was expressed, and discs were assayed for mislocalization of the nuclei and centrosomes in dividing mutant cells.

This chapter demonstrates that loss of Gli in columnar epithelia results in cell lethality and basal mislocalization of the plane of cell division. The SJs are also required to maintain the plane of cell division, but through the localization of Gli to the TCJ.
Materials and Methods

Fly Strains

Stocks referred to as wildtype are w^1118 or the GFP fusion protein lines outlined below. To down-regulate the SJ core proteins in imaginal wing discs we used the following UAS RNAi lines from the Vienna stock centre (Dietzl et al., 2007): UAS-cora RNAi (VDRC 9787 & 9788), UAS-Nrg RNAi (VDRC 6688 & 27201), UAS-NrxIV RNAi (VDRC 8353 & 9039) and UAS-Gli RNAi (VDRC107258); and UAS-Gli RNAi (NIG 3903R-3) from NIG-FLY stock centre (Hoskins et al., 2007). We used the apterous-GAL4 (Bl: 106798) insertion line from the Bloomington stock center. UAS-Dicer 2 (Dietzl et al., 2007) was co-expressed with RNAi lines to increase RNAi efficiency and was obtained from the Bloomington stock center (Bl: 24664).

For wild type analysis of cell division, we used GFP fusion proteins from the Bloomington stock center or the Fly Trap Project, Dlg-GFP (CC01936), Zip-GFP (CC01626) (Buszczak et al., 2007) and Jupiter-GFP (G00147), Nrg-GFP (G00305), (Morin et al., 2001).

Immunofluorescence

Immunolabeling of third instar imaginal wing discs was carried out as described previously (Schulte et al., 2006). All images were generated on a Deltavision Restoration microscope (Applied Precision). Data was collected using a 60X(1.4 NA) oil immersion lens using a CoolSnap HQ digital camera. Data from all wavelengths was collected for each 0.2 um optical section before the next section was collected. SoftWorx (Applied Precision) software was used for deconvolution of 8-10 iterations using a point spread function calculated with 0.2 um beads conjugated with Alexa Fluor 568, 488 and 647 (Molecular Probes) mounted in Vectashield. Images were then exported to Photoshop CS4 for generation of figures.
Primary antibodies used in study were: mouse anti-Dlg 4F3 at 1:300 (Parnas et al., 2001), rat anti-DE-cadherin at 1:50 (Oda et al., 1994), mouse anti-Coracle (9C and C615-16B cocktail) at 1:500 each (Fehon et al., 1994), mouse anti-Gliotactin 1F6.3 at 1:100 (Auld et al., 1995) or rabbit anti-Gliotactin at 1:300 (Venema et al., 2004), rabbit anti-Anillin 1:600 (Goldbach et al., 2010), mouse anti-γ-tubulin 1:600 (Abcam), mouse anti-acetylated-tubulin 1:600 (Abcam), mouse anti-α-tubulin 1:600 (Sigma), and rat anti-tubulin 1:600 (clone YL1/2 Millipore), rabbit anti-phospho-Histone 3 1:600 (Abcam), mouse anti-Peanut 1:100 (Neufeld and Rubin, 1994), mouse anti-Kinesin 1:100 (Ingold et al., 1988). All the following secondary antibodies were used at 1:200 dilution: goat anti-mouse, anti-rabbit or anti-rat conjugated to one of Alexa Fluor 488, Alexa Flour 568 and Alexa Fluor 647 or Cy5, (Molecular Probes). Nuclei were detected with DAPI at 1:1000.

**Ectopic expression of RNAi and assaying mitotic stages**

Crosses of individual RNAi lines were crossed to the apterous-GAL4 driver at progeny raised at 25°C or 29°C. Imaginal discs were isolated from wandering 3rd instar larvae and fixed following standard protocols (Schulte et al., 2006). Antibody stains were repeated a minimum of twice and 10 imaginal discs were imaged for each experiment to control for consistency. Discs that were damaged or otherwise varied from other wildtype samples were excluded.

For assaying the stage of mitosis, the areas assayed were exclusively in the wing pouch and images were collected using a 1024X1024 pixel area with a 60X objective (122X122 microns). Images were collected across the apterous dorsal/ventral border and thus for each region assayed, one half was composed of wild-type cells and the other half was apterous-GAL4 expressing cells. Each stage of mitosis was assayed as follows: metaphase and anaphase with DAPI, γ-
tubulin, phospho-Histone3 immunofluorescence; telophase A-C with Dlg, \( \gamma \)-tubulin, DAPI immunofluorescence.

**Generation of Gliotactin null clones**

The Gli\(^{dv3} \) FRT40A chromosome was generated previously through recombination (Venema et al., 2004). Clones were generated using mosaic analysis with a repressible marker (MARCM) (Lee and Luo, 2001). Gli\(^{dv3} \), FRT40A/Cyo; 69B GAL4 males were crossed to UAS Flipase/UAS Flipase; UAS mCD8GFP FRT40A females. The F1 third instar larvae were heat-shocked at 37\(^\circ\) C for 3 hours, 24 hours before dissection, to increase the efficiency of the Flipase. Only clones express UAS mCD8GFP construct under the control of 69B GAL4, an ubiquitous imaginal wing disc driver (Brand and Perrimon, 1993; Brand et al., 1994). After fixation and immunofluorescence labelling, all mutant cells are marked with membrane localized mCD8-GFP. Clone presence was further confirmed with immunolabeling for Gliotactin to ensure absence of the protein in cells.
**Results**

**Gliotactin null clones are cell lethal and fail to undergo cell division**

Gli null clones generated in the imaginal wing disc are small, cell lethal (Schulte et al., 2003; Venema et al., 2004), and have no discernable effect on the SJ core proteins or apical/basal polarity (Schulte et al., 2006). To further investigate Gli-/- cells and determine what caused the lethality, the MARCM system with mCD8-GFP was used to follow and identify clones in the wing pouch (Figure 3.1A-F) and immunolabeling for Gli confirmed the Gli genotype (Figure 3.1A, arrowhead). As expected, the apical membrane was intact, as shown by immunolabeling with the adherens junction marker, phosphorylated Src (Figure 3.1A, arrowhead). On further investigation an accumulation of pyknotic nuclei was noticed on the basal side of the epithelia (Figure 3.1B, arrowheads), strongly suggesting cells mutant for Gli were undergoing apoptosis and delaminating. Apoptosis was confirmed by showing that Gli null clones that had delaminated were positive for activated Caspase 3 (Figure 3.1F, arrowhead).

As the SJ proteins are not affected and apical/basal polarity is maintained, there was not an obvious explanation for the cell lethality seen in Gli null clones (Schulte et al., 2003). One observation was that Gli null clones delaminated and underwent apoptosis in random clusters. A cellular process known to occur in a pseudo-random pattern in the imaginal wing disc is cell division (Gonzalez-Gaitan et al., 1994), suggesting that Gli could be involved in cell division and undergoing apoptosis due to a disruption in this process.

Mitotic cells were assayed for in Gli null clones by immunolabeling with PH3 and DAPI. Mitotic profiles were rare, and when mitotic null clones were observed, they were always bordered by wildtype cells (Figure 3.1A, arrow, D-E, arrowheads), suggesting that neighboring wildtype cells rescued the ability of mutant cells to undergo cell division. Observations suggest
that a clone had to be bordered by at least two wildtype cells in order for cell division to be rescued. Added to this was the rarity of large, multi-cellular clones; the larger clones shown in Figure 1 were rare events and the majority of Gli null clones found were 1-4 cells in size with all mutant cells contacting wildtype neighbors.

Since the absence of Gli resulted in cell lethality, and there appears to be a disruption in cell division, the next question to ask was whether a disruption in the SJs core proteins had a similar effect on cell division in columnar epithelial cells, as clones for the SJ core complex also show a similar cell lethal phenotype.

**Downregulation of the SJs leads to a mislocalization of the plane of cell division**

Since cell division is localized to the SJ domain, discs where the core SJ components, Cor, Nrx IV, and Nrg, had been disrupted were assayed for a mislocalization of the plane of cell division or an absence of dividing cells. To disrupt the SJs, RNAi was expressed for Nrg (Figure 3.2A), Cor (Figure 3.2B), and NrxIV (Figure 3.2C-F) in specific regions of the wing imaginal disc using the GAL4 drivers patched (Figure 3.2A) and apterous (Figure 3.2B-F). This allowed many mutant and wildtype cells to be assayed side by side and overcame the difficulty of assaying small SJ null clones. To assess that the SJs were disrupted, cells were immunolabeled for the septate junction protein Coracle, which is always mislocalized in Nrg and NrxIV mutants (Genova and Fehon, 2003).

In Nrg, NrxIV and Cor RNAi expressing cells, Cor was downregulated and mislocalized basally (Figure 3.2A, arrow) or absent (Figure 3.2B,D, arrows), and E-cadherin was not affected (Figure 3.2C, arrow), confirming that the SJs were disrupted and apical/basal polarity was intact. There was a dramatic, basal mislocalization of Gli (Figure 3.2A-C, arrowheads), where Gli was
no longer localized to the TCJ (Figure 3.2E, arrowheads) (Schulte et al., 2003), but spread around the perimeter of the cell as well as basolaterally (Figure 3.2F, arrowhead). In NrxIV RNAi expressing discs, Dlg was also somewhat downregulated (Figure 3.2G, arrow), but not as dramatically as Cor.

Imaginal discs expressing NrxIV RNAi under the control of apterous GAL4 were next assayed for a mislocalization of the centrosome marker γ-tubulin and mitotic DNA marker PH3. γ-tubulin was mislocalized basally in dividing cells (Figure 3.3A, arrows). Using DAPI to assay for mitotic stage, it was determined that the basolateral spread of dividing cells was not specific to any one stage (Figure 3.3B-E). This means that in absence of the SJs, the plane of cell division can occur far below the normal range in wildtype cells.

To assay the basal spread of the plane of cell division, the distance the centrosomes migrated basally was quantified and compared to the average distance of 2.6 microns below the adherens junctions that the plane of cell division is found in wildtype cells. This was determined by averaging the maximum distance between the adherens junctions and the more basal centrosomes in dividing wildtype cells (n=5 imaginal discs/n=328 centrosomes). In NrxIV RNAi cells, the centrosomes were found on average 6.93 microns basal to the adherens junctions, with a maximum range of 11.8 microns (n=3 discs/n=59 centrosomes). The overall number of centrosomes in wildtype cells found below the plane of cell division on average was 2.6, compared to 24.6 centrosomes found within the normal plane of cell division (n=5 discs). In NrxIV RNAi cells, an average of 11 centrosomes were found within the normal plane of cell division and 34.4 centrosomes below (n=5 discs). These results show that a disruption in the SJs leads to basal mislocalization of the plane of cell division.
Of note, the distance that the centrosome spread correlated with the basal spread of Gli (Figure 3.3A, Gli). It was next asked whether disruption of Gli would lead to mislocalization of the plane of cell division as well.

The spindle is “anchored” at a tricellular junction in columnar epithelial cells through localization of one of the centrosomes

To determine if Gli was involved in localizing the plane of division in columnar epithelia, centrosomes were first assayed to determine if they showed any preferential localization or relationship to the TCJ in wildtype columnar epithelia. The plane of cell division never bisects the TCJ in columnar epithelial cells (Gibson et al., 2006), suggesting that one of the centrosomes is localized at or near the TCJ. The localization of the centrosomes in relation to Gli was determined in dividing prophase to telophase A cells (Figure 3.4A-E, arrowheads). In wildtype columnar epithelia, one of the centrosomes, immunolabeled with γ-tubulin, is always associated with the TCJ (n= 10 discs, 20 metaphase centrosome pairs), immunolabeled by Gli. However the centrosomes do not directly co-localize with Gli (Figure 3.4A-C, arrowhead).

The association of only one of the centrosomes with the TCJ can be explained by the variation in number of cell sides in the imaginal disc (Gibson et al., 2006). Only a six-sided cell would show both centrosomes at the TCJ, while a five-sided cell would only have a single centrosome orientated to the TCJ. This localization persists from prophase through anaphase (Figure 3.4C, arrowheads). At telophase A, the centrosomes begin to migrate back to the apical side of the cell where they localize during interphase, thus exiting the SJ domain/TCJ region (Figure 3.4D-E, arrowheads).
The localization of at least one of the centrosomes to the TCJ in columnar epithelia demonstrated a preferential localization, so discs where Gli had been downregulated were next assayed for a mislocalization of the centrosomes as well as a basal mislocalization of the plane of cell division.

**Gliotactin maintains the plane of cell division**

To determine whether Gliotactin was required for the localization of the centrosomes to the TCJ, imaginal wing discs expressing Gli RNAi were assayed. In dividing cells, the spindle still formed properly (data not shown), and when spindle localization was looked at with respect to the SJ marker Dlg, found concentrated at the TCJ (Schulte et al., 2003), the centrosomes are still localizing to the TCJ (data not shown). However, without the TCJ marker Gliotactin, it is difficult to tell. It was concluded that Gli is not required for association of the centrosomes with the TCJ and is not involved in spindle orientation.

Given the spread of Gli in the SJ RNAi mutants, imaginal discs expressing Gli RNAi under the control of apterous-GAL4 were assayed, and showed a dramatic, basal mislocalization of the centrosomes (Figure 3.5A-B, arrowheads). In Gli RNAi expressing discs (n=5), on average 21.6 centrosomes were found within the plane of cell division on the wildtype side and only 3.3 centrosomes were found below the plane of cell division. On the mutant side 21.6 centrosomes were found below the plane of cell division and only 8.6 were found at the plane of cell division. No centrosomes were found basal to the range determined in the NrxIV RNAi experiment (11.6 microns below the adherens junctions). Those mitotic cells that had fallen below the normal SJ domain were next looked at to determine if the basal mislocalization was specific to a mitotic stage. Cells below the wildtype plane of division were found up to late anaphase when assayed
with DAPI and γ-tubulin (Figure 3.5C-D). Unfortunately all telophase markers used here are based on SJ proteins and as these cells were below the SJ domain it was not possible to test for stages beyond late anaphase.

In wildtype cells, at the end of telophase A, the centrosomes begin an apical migration that continues through telophase C (Figure 3.4G), until the centrosomes reach their normal interphase location at the apical side of the cell (Figure 3.4H-J). During interphase, the centrosomes remain at the apical side of the cell but are less condensed (Figure 3.5A, left side γ-tub). In Gli RNAi expressing cells it was observed that the γ-tubulin normally associated with the apical side of the cell at interphase was significantly downregulated (Figure 3.5A, right side), demonstrating that Gli is necessary to maintain the plane of cell division at the SJ domain and maintain γ-tubulin at the apical side of the cell.

**Maintenance of the Plane of cell division is specific to Gliotactin**

It was next asked whether the mislocalized plane of cell division seen in SJ RNAi and Gli RNAi was specific and unique to a downregulation of Gli, or due to a disruption of the SJs in general. A SJ associated protein, Discs-large (Dlg), was also downregulated as a comparison, due to its association with the SJs and genetic interactions with Gli (Schulte et al., 2006), as well as the role the DLG complex plays establishing apical/basal polarity in the embryo (Bilder, 2001).

When RNAi for Dlg was expressed using apterous-GAL4, Gli was downregulated along with Dlg (Figure 3.6A-D), but the adherens junctions and SJ domain remain intact (Figure 3.6B,D). Although the Dlg epitope was strongly reduced in wing disc epithelium (Figure 3.6A), it did not lead to a loss of polarity. Either the downregulation of Dlg is not strong enough to elicit an effect on apical/basal polarity, or that because not all isoforms of Dlg are targeted, only those
necessary for the localization of Gli are affected. In some discs where the downregulation of Dlg was weaker, Gli levels were reduced but not mislocalized basally (Figure 6C, arrowheads) or around the cell (data not shown), as was seen in the SJ RNAi mutants. This indicates that Dlg is necessary for maintaining Gli at the TCJ, but not the adherens or septate junctions. Conversely, the SJs are necessary for restricting Gli to the TCJ and at the SJ domain (Figure 3.2). When the plane of cell division was assayed, \( \gamma \)-tubulin was mislocalized in Dlg RNAi expressing cells (Figure 3.6B, arrowheads). The mislocalization of \( \gamma \)-tubulin correlated to the region where Gli was reduced (Figure 3.6B). In discs where the down-regulation of Gli was not fully penetrant, \( \gamma \)-tubulin immunolabeling indicates that cell division is localized normally within the plane of cell division (Figure 6C, arrowheads). It was also observed that the SJs, as shown by immunolabeling Cor were not disrupted (Figure 3.6D). This again suggests that the mislocalization of the plane of cell division seen in Gli RNAi, SJ RNAi, and Dlg RNAi expressing cells is specific to the absence or mislocalization of Gli.

A second mitotic marker, Anillin (Chapter 2), which labels the contractile ring, was also looked at to determine whether the contractile ring was mislocalized in a Gli RNAi background. Although Anillin was not mislocalized in Gli RNAi expressing cells, it was never found below the SJs in mutant cells (Figure 3.7E-F, arrowheads). This suggests that although the plane of cell division mislocalizes basally in cells where Gli has been down-regulated, the contractile ring is absent or unable to assemble in dividing cells below the SJ domain. It appears as if Anillin and the contractile ring can still form in mutant cells, provided the dividing cell falls within the SJ domain. This suggests that in the absence of Gli, the plane of cell division becomes randomly distributed and when the random distribution coincides with the SJ domain, the contractile ring
assembles. This also suggests that there could still be another role for the SJs later in cytokinesis concerning the assembly or the contractile ring, which is investigated in Chapter 4.

**Downregulation of Gliotactin does not disrupt the SJs but does disrupt the microtubule cytoskeleton**

To address whether the downregulation of Gli had an effect on the localization of the SJs or Dlg in columnar epithelia, Gli RNAi expressing cells were immunolabeled for Dlg and Cor. Prior observations in Gli somatic null clones suggested that the loss of Gli did not result in changes to Dlg or the SJs (Schulte et al., 2006). Similarly, when cells were immunolabeled for Dlg in a Gli RNAi background, it was not affected (Figure 3.7A, arrow). Cor was still localized to the SJs in a Gli RNAi background, even though the levels of both junctional and non-junctional Cor were slightly down-regulated (Figure 3.7C, Cor).

One possibility for the mislocalized plane of division in cells where Gli had been downregulated is that the mechanisms or structures responsible for nuclear migration are disrupted. When wildtype cells were immunolabeled for microtubules, a high concentration of acetylated tubulin was observed at the TCJ basal to the normal location of Gli (Figure 3.7G, arrow). However, immunolabeling for acetylated tubulin was affected in the Gli RNAi expressing cells (Figure 3.7C, arrow). Acetylated tubulin is normally found distributed through the entire height of the cell (Figure 3.7C-D, left of arrow), but in the Gli RNAi expressing cells, acetylated tubulin is downregulated in the region below the SJs (Figure 3.7C, arrow).

The motor proteins Dynein and Kinesin are necessary for nuclear migration (Baye and Link, 2008) though the exact mechanism is undetermined. Nrx IV RNAi expressing cells were tested for the distribution of the motor protein Kinesin in NrxIV RNAi, as it is known to be involved in
interkinetic nuclear migration in *C. elegans* and centrosome migration in *Drosophila* (Fan et al., 2004; Fridolfsson and Starr, 2010) Kinesin immunolabeling was found throughout the full extent of the microtubule network (Figure 3.7D) in wildtype cells and extended from the basal to apical sides of the epithelium. Not unexpectedly, Kinesin was also downregulated to the same extent as the microtubules in the *Nrx IV* RNAi cells (Figure 3.7D). These results suggest that the loss of Gli or the mislocalization of Gli results in a disruption of the underlying microtubule network, providing a potential mechanism through which Gli could be affecting centrosome and nuclear migration during cell division.
**Discussion**

Cell division in columnar epithelia of the imaginal wing disc occurs at the level of the SJs, (Gibson et al., 2006) and a SJ associated protein, Gli, is necessary to maintain this plane of cell division. Downregulation of Gli, either directly or through the downregulation of NrxIV or Dlg, leads to a plane of division that is no longer maintained at the correct level and spreads basally within the epithelium. As Gliotactin is a TCJ protein, this also implies a role for the TCJ in maintaining this localization. The fact that neither the SJs nor Dlg were mislocalized in a Gli mutant background suggests that Gli, or another as yet unidentified component at the TCJ, is solely responsible for this positioning.

A possible mechanism through which Gli and the TCJ could be affecting the plane of cell division is through nuclear migration, or more specifically, through an effect on the microtubule cytoskeleton. Acetylated tubulin is found concentrated at the tricellular corner and extends from the base of the epithelia to the base of the TCJ. Downregulation of Gliotactin leads to a a disorganization of the microtubule network under the TCJ disruption leading us to propose a model in which Gliotactin is involved in the apical translocation of the nucleus during cell division through the maintenance of the microtubule network and the potential control of the microtubule associated motor proteins.

**Conserved mechanisms for apical nuclear migration during mitosis**

The movement of the nuclei in the wing imaginal disc appears to be analogous to the interkinetic nuclear migration seen in neuroepithelia. In neuroepithelia, DNA replication (S phase) occurs at the basal surface of the cell. The nucleus then migrates within the cytoplasm to properly position itself within the apical/basal plane for mitosis (M phase) and cytokinesis (Frade, 2002; Baye and
Microtubule-based motor proteins and specific nuclear envelope proteins are essential for the process of nuclear migration and positioning in neuroepithelia (Morris, 2003; Xiang and Fischer, 2004; Starr and Fischer, 2005; Wilhelmsen et al., 2006).

Overall, studies in multiple organisms have strongly implicated two microtubule motor proteins that act to translocate the nucleus; Dynein and Kinesin (Baye and Link, 2008). Dynein is a multi-protein, minus-end directed microtubule motor and Kinesin is the plus-end directed microtubule motor (Whited et al., 2004). Dynein is responsible for apical (minus end) movement of the nuclei along the microtubules, and Kinesin is responsible for the basal movement (minus end). It is a shift in the balance between these two opposing forces that lead to migration in either direction (Baye and Link, 2008; Fridolfsson and Starr, 2010). For instance, in the Drosophila eye imaginal disc, a disruption of Dynein effects apical movement of the nucleus (Fan and Ready, 1997), and in nematodes Kinesin is necessary for migration of the nucleus back to the basal side of the cell at the end of cytokinesis (Baye and Link, 2008; Fridolfsson and Starr, 2010).

Dynactin is another multi-protein complex that is necessary to activate Dynein and promote processivity between the motor and microtubules (Schroer, 2004). The nuclear positioning phenotypes caused by mutations in Dynein or Dynactin can be suppressed by mutations in Kinesin, which implicates that Kinesin family members likely play a role in balancing the minus-end directed forces of Dynein-dynactin, and vice versa (Baye and Link, 2008).

Kinesin and Dynein act to direct movement along the microtubules, but how are they linked to the nuclear envelope? One way is through Dynactin, which is also required for the binding of cargo to Dynein (Schroer, 2004). Although not definitive, it is thought that Dynactin interacts with the KASH domain of Klarsicht, a perinuclear membrane associating protein that shares a C-
terminal domain with ANC-1 of C. elegans and Syne/Nesprin proteins of vertebrates (Starr and Fischer, 2005; Wilhelmsen et al., 2006; Baye and Link, 2008). Mutations in the KASH domain proteins have been shown to disrupt nuclear positioning in both vertebrates and invertebrates suggesting that the KASH-domain group of proteins link the nuclear envelope to the cytoskeleton (Apel et al., 2000; Starr and Han, 2002; Grady et al., 2005). The third group of nuclear migration components is the SUN domain proteins which are KASH-protein receptors localized to the nuclear membrane (Worman and Gundersen, 2006). SUN-domain proteins are found in all eukaryotes and are important for nuclear envelope morphology by linking the outer and inner nuclear membranes to the nuclear lamina (Crisp et al., 2006). It is likely that Kinesin also associates with the nucleus through the same or similar nuclear membrane proteins (Baye and Link, 2008).

Microtubules extend from the basal to apical surface in imaginal disc epithelia (Fristrom, 1976; Eaton et al., 1996; Fan and Ready, 1997). The distribution of the microtubules under the TCJ in the wing epithelium suggests a role for Gliotactin and the TCJ in microtubule-based motility in nuclear migration. The failure of Gliotactin null clones to undergo mitosis in absence of wildtype neighbours, paired with the downregulation of the cytoskeleton components acetylated-tubulin and Kinesin raises an interesting possibility; that Gliotactin is necessary for the apical migration of the nucleus, as well as the localization of the plane of cell division to the SJs.
Gliotactin is necessary for localizing the plane of cell division to the septate junction domain

In wing columnar epithelia, the microtubules are oriented with their minus ends toward the apical cell surface and the plus ends at the basal side (Mogensen et al., 1989), an orientation that is conserved in vertebrate epithelia (Bacallao et al., 1989), and therefore suggests a conserved directional movement of Kinesin and Dynein. If the two motor proteins are necessary for the migration of the nuclei, then a disruption of Kinesin and/or the microtubules, as seen in Nrx IV and Gli RNAi expressing cells (Figure 3.7C,D, G-H), would be expected to lead to failure of proper nuclear migration and possibly cell division. Unfortunately, the assay for Dynein mislocalization was inconclusive (data not shown).

Regardless, a disruption of Gliotactin leads to a dramatic effect on the plane of cell division, where not all cells reach the plane of cell division, as shown by the centrosome marker, γ-tubulin (Figure 3.6B). However, is localization of the plane of cell division to the septate junctions necessary for cell division to proceed? The failure of Anillin to assemble below the septate junction domain in a Gli RNAi background, suggests that the contractile ring is not able to form when the plane of cell division is localized below the SJ domain. This further suggests that only those cells with a plane of division that has randomly localized within the SJ domain form a contractile ring and undergo cytokinesis. Those cells with a plane of division below the SJ domain are able to reach late anaphase (Figure 5C-D), but in the absence of the SJs fail to enter telophase and form the contractile ring. This also raises an interesting question concerning the viability of a cell that attempts to divide without a fully formed contractile ring. The absence of Anillin from cells dividing below the SJ domain, should result in an inability to complete cytokinesis and likely is the cause of the cell lethality observed in the Gli RNAi cells.
A second barrier below the septate junctions

As the mislocalized dividing cells never reach the basal most side of the cell, it is possible that there is another domain below the SJs that prevents the complete basal spread of components in the absence of Gli. Both the Gli RNAi and Dlg RNAi support this hypothesis. The microtubule cytoskeleton showed a concentrated pattern at the TCJ basal to the SJs and Gli. In Gli and NrxIV RNAi expressing cells, the microtubules are downregulated in the region directly below the SJs but persist at the basal side of the cell and no longer hold their organized tricellular pattern (Figure 3.7H). The motor protein Kinesin was also downregulated alongside the microtubules (Figure 3.7D,G-H), however it was not possible to assay for Dynein with available antibodies (data not shown). It is possible that either the motor proteins or microtubule network itself is disrupted to an extent where the nucleus cannot orientate itself to the SJs. Those nuclei that do reach the SJ domain undergo cytokinesis, which explains the properly forming contractile ring at the plane of cell division (Figure 3.7E-F). The nuclei that fail to reach the SJs would then not form a contractile ring and fail to undergo cytokinesis.

Gliotactin is necessary for the apical localization of γ-tubulin during interphase

As discussed in chapter 2, γ-tubulin is a marker for the centrosomes during cell division and localizes to the apical side of the cell during interphase. The movement of the centrosome marker γ-tubulin from the apical side of the cell, to the SJs and presumably back to the apical side of the cell parallels what has been shown for centrioles in vertebrate epithelial cells (Reinsch and Karsenti, 1994; Baye and Link, 2007; Schenk et al., 2009). Although this parallel suggests a conserved mechanism, Drosophila epithelia do not have centrioles (Basto et al., 2006).
However, instead of centrioles, it is possible that Drosophila have a centriole-like structure that is localized to the apical side of the cell and incorporates γ-tubulin.

An interesting observation was the reduction in the γ-tubulin immunolabeling at the apical side of interphase cells in Gli RNAi and Dlg RNAi expressing cells. As this phenotype was not seen in NrxIV RNAi expressing cells, the localization of γ-tubulin at the apical side of the cell is likely correlated to the presence or absence of Gli and not its localization. The reduced levels of γ-tubulin at the normal interphase location may be a reflection of the changes in the microtubule cytoskeleton or a failure to return the centriole–like structure to the apical side of the cell during late cytokinesis.

Conclusion
An interesting role has been uncovered for a Drosophila junctional protein, raising the possibility that analogous vertebrate structures may be used for the same purpose. Cell division in vertebrate epithelia occurs at the vertebrate tight junctions (Reinsch and Karsenti, 1994) and Tricellulin is found at vertebrate TCJs similar to Gli (Ikenouchi et al., 2005). It is possible that Tricellulin, or another vertebrate TJ protein, is involved in maintaining the plane of cell division and/or nuclear migration. Further analysis of cell division in columnar epithelial will continue to uncover proteins and complexes involved in maintaining cell division in a three-dimensionally structured cell, and may uncover more potential vertebrate candidates.
**Figures**

**Figure 3.1 Null clones for Gliotactin fail to undergo cell division**

A) Somatic null clones for the Gliotactin null allele (Gli\textsuperscript{dv3}) using MARCM (FRT Flipase mediated recombination). Null clones are marked with mCD8-GFP. Activated Src (pSrc, green) marks the adherens junctions and is not affected in Gliotactin null cells (Gli, red; arrowhead). The null clone is identified by the absence of Gliotactin (arrowhead), and the presence of mCD8-GFP (blue).

B) Pyknotic nuclei (DAPI, blue; arrowheads) mark the cells that have delaminated at the basal side of the epithelia. The basal side of the cells are marked by \(\alpha\)-tubulin (red, arrows). Gli null cells are marked by mCD8-GFP (green).

C) Apical en face view of the clone seen in B. The absence of Gliotactin (Gli, red) and presence of mCD8GFP marks the clones (green, arrow). The \(\alpha\)-tubulin (blue) pattern indicates the apical side of the cell.

D-E) A dividing Gliotactin null cell immunolabeled with \(\gamma\)-tubulin (red) at telophase A (arrowhead), surrounded by wildtype cells. D shows en face and E shows a side projection of the same cell. The star denotes neighboring, delaminating Gliotactin null cells marked with mCD8GFP (green).

F) Delaminating Gli\textsuperscript{dv3} clones are apoptotic as shown by immunolabeling of activated Caspase 3 (red, arrowhead) in basally located cells (z=75). Gliotactin immunolabeling (Gli) shown at level of SJ (z=10) demonstrate that the wildtype membrane has sealed over the mCD8GFP marked null cells (green).

G) Cartoon diagram indicating the apical/basal location of the cells shown in A-F. Clones = Green, apical membrane = yellow, SJs = red, nuclei = blue, centrosomes = black.
Figure 3.2 Expression of SJ RNAi in the imaginal wing disc leads to a disruption in Gliotactin

A) Expression of Nrg RNAi using the patched-GAL4 driver. Cora (red) is down-regulated (arrow) and Gliotactin (Gli, green), is mislocalized basally (arrowhead) in a discrete region of the wing disc.

B) Expression of Cora RNAi using the apterous-GAL4 driver. The arrowhead indicates the border between wildtype (right) and mutant (left) tissue. Cora (red) is absent on the apterous GAL4 side (arrow). Gliotactin (Gli, green) is mislocalized basally (arrowhead).

C) Expression of Nrxi IV RNAi using the apterous-GAL4 driver. The apical membrane and polarity of the cell is intact as shown by the normal localization of E-cadherin (red; arrow). Gliotactin (Gli, green) is mislocalized basally (arrowhead).

D) Expression of Nrxi IV RNAi using the apterous-GAL4 driver. Cora (green) is absent in this region (right of the arrowhead).

E-F) En face view of wildtype columnar epithelia (E) and Nrxi IV RNAi expressing columnar epithelia (F) at the level of the SJ immunolabeled with Dlg (red). Gliotactin is no longer restricted to the TCJ in F (Gli, green, arrow).

G) Expression of Nrxi IV RNAi using the apterous-GAL4 driver. Dlg (red) is downregulated on the apterous side (right of arrow). Arrowhead indicates delaminating pyknotic nuclei (DAPI, blue).

Each scale bar represents 15 microns.
Figure 3.3 Expression of SJ RNAi leads to a dramatic mislocalization of the plane of cell division

3rd instar imaginal discs expressing NrxIV RNAi with apterous-GAL4.

A) Side projection of a separate mutant wing disc shown at a higher magnification across the apical region (~10 μm) with mislocalized Gliotactin (Gli, green) marking the mutant side. Large arrows on either side of the panels indicate the most basal range of centrosomes immunolabeled with γ-tubulin (G-Tub, red) during cell division in wildtype cells. Small arrows indicate those centrosomes on the mutant side located below the wildtype plane of cell division.

B-E) Mitotic cells in NrxIV RNAi cells, basal to the wildtype plane of cell division can be found in all stages of mitosis up to late anaphase. Gliotactin (Gli, green) was used to mark the cell boundaries, γ-tubulin (γ-tub, red) marks the centrosomes and DAPI (blue) marks DNA and the mitotic stage. B) metaphase, C) early anaphase D) anaphase, E) late anaphase/early telophase.
Figure 3.4 The centrosomes associate with the tricellular corner

Cells from 3rd instar imaginal wing discs were imaged at the level of the SJs and immunolabeled for the centrosomes (γ-tubulin, G-Tub, blue), the septate junctions (Nrg-GFP, red) and the tricellular junction (Gliotactin, Gli, green).

A) At prophase both centrosomes have already localized to opposite side of the cell, and one is associated with the tricellular corner (TCJ) as shown by Gliotactin (arrowhead).

B) By metaphase, the cell diameter has increased and one of the centrosomes is still localized to the TCJ (arrowhead).

C) At anaphase the association of one of the centrosomes with one of the TCJs is still visible (arrowhead).

D-F) At the onset of telophase A, the ingression furrow forms and dislocates the centrosomes from their association with the TCJ. D shows the apical region (z52) of the SJs in a cell at early telophase. E shows the basal region of the SJ domain of the same cell (z67). Arrows indicate the ingression furrow, arrowheads indicate centrosomes. F shows a side projection of the telophase A cell in D and E and indicates the two regions where the enface images were taken from.

G-J) By late telophase A the centrosomes are still located within the SJ domain. H) By telophase B the centrosomes have begun to migrate apically. I-J) This process continues through telophase C (I), until the centrosomes are localized to the apical most side of the cell (J).

Inset cartoons are illustrations of the adjacent left panel.
Figure 3.5 Expression of Gliotactin RNAi leads to a mislocalization of the plane of cell division

3\textsuperscript{rd} instar imaginal discs ectopically expressing Gli RNAi with apterous-GAL4.

A) Side projection across the entire height of Gliotactin RNAi (right of arrowhead) and wildtype (left of arrowhead) columnar epithelial cells. The absence of Gliotactin (Gli, green) indicates Gliotactin is down-regulated. Apically localized $\gamma$-tubulin ($\gamma$-tub, red) is down-regulated on the mutant side.

B) Side projection of a separate mutant wing disc shown at a higher magnification across a greater number of z slices. The absence of Gliotactin (Gli, green) indicates the mutant side. Arrowheads indicate those centrosomes immunolabeled with $\gamma$-tubulin ($\gamma$-tub, red) on the mutant side located below the wildtype plane of cell division.

C-D) Mitotic cells basal to the wildtype plane of cell division in Gliotactin RNAi discs, at C) metaphase and D) late anaphase. $\gamma$-tubulin ($\gamma$-tub, red) marks the centrosomes and DAPI (blue) marks DNA and the mitotic stage.

E) Gliotactin RNAi cells immunolabelled for Gliotactin (Gli, green) and phosphorylated Histone 3 (PH3, red). The nuclei in dividing cells on the mutant side fall below the normal plane of cell division (arrow).

Scale bars represent 15 and 5 microns as indicated in figures. All enface panels represent a single Z slice of 0.2 microns.
Figure 3.6 Mislocalization of the plane of cell division is associated with the mislocalization of Gliotactin

3rd instar imaginal discs expressing Dlg RNAi with apterous-GAL4 (A-D). A) Side projection of Dlg RNAi expressing cells. The arrow indicates the boundary between wildtype (left) and Dlg RNAi expressing cells (right). Both Dlg (red) and Gliotactin (Gli, green) are strongly downregulated.

B) Side projection across Dlg RNAi (right) and wildtype (left) expressing cells. Gliotactin (Gli, green) is down-regulated and γ-tub marking the mitotic centrosomes (red) is mislocalized basally on the mutant side (arrowhead). Immunolabeling for E-cadherin shows the adherens junctions are intact indicating the cells have not lost apical/basal polarity.

C) Side projection of a separate mutant wing disc where some Gliotactin was left at the membrane. Some Gliotactin immunolabeling remains (Gli, green, arrowhead) and the centrosomes immunolabeled with γ-tubulin (red, arrowhead) are not mislocalized.

D) The SJs immunolabeled with Cora (red) and the adherens junctions immunolabeled with E-cadherin (blue) are not disrupted in the area where Dlg RNAi is expressed as shown by the absence of Gliotactin (green).

Scale bars represent 15 and 5 microns as indicated in each panel.
Figure 3.7 Downregulation of Gliotactin leads to downregulation of acetylated-tubulins in the cytoskeleton.

3rd instar imaginal discs expressing Gliotactin RNAi with apterous-GAL4.

A-B) Side projection across Gliotactin RNAi (right of arrowhead) and wildtype (left of arrowhead) cells. Mutant cells are indicated by absence of Gli. Dlg and Ecad (red) are not mislocalized when Gliotactin is downregulated (green).

C) Side projection across Gliotactin RNAi and wildtype cells. The arrow indicates the border between mutant (right) and wildtype (left). Junctional and non-junctional Cora (green) are down-regulated but not mislocalized from the SJs. Acetylated tubulin is also down-regulated (red) and mislocalized. Delaminated pyknotic nuclei are indicated (arrowheads).

D) NrxIV RNAi expressing cells immunolabeled for Gliotactin (green), α-tubulin (red) and Kinesin (blue) or DAPI (blue). α-tubulin and Kinesin are downregulated basal to the SJs when the SJs and Gliotactin are disrupted (arrows).

E-F) Side projections across Gliotactin RNAi and wildtype cells. Arrowheads indicate contractile rings immunolabeled for Anillin (red) forming within the SJ domain on the mutant side. Cells were also immunolabeled for Gliotactin (green) and E-cadherin (Blue).

G) Enface view showing the concentration of acetylated tubulin to the tricellular corner basal to the TCJ (arrowheads) (z=54).

H) Enface view showing the disruption in tricellular acetylated tubulin in Gli RNAi expressing cells (arrowheads) (z=50).

All scale bars represent 15 microns.
IV. The septate junctions are necessary for the completion of cytokinesis

Introduction

In Drosophila epithelia the septate junctions (SJs) and tricellular junctions (TCJs) function primarily as paracellular diffusion barriers, establishing and maintaining permeability barriers in a variety of epithelial tissues, including the glia and salivary glands (Tepass and Hartenstein, 1994; Lamb et al., 1998; Tepass et al., 2001). The SJs occupy the apical-lateral region of the membrane directly below the adherens junctions and are composed of an electron dense material, called septae, that are spaced uniformly between cell membranes. The organization and uniform alignment of the septae between adjacent cells give the SJ the appearance of a ladder with rungs (Tepass and Hartenstein, 1994). At the corners of epithelia where three cells meet, the SJs turn and run perpendicular to the cell to form the tricellular junction (TCJ). The core of the TCJ is sealed together by the tricellular plug, electron dense material that forms evenly spaced plugs between the adjacent cells and functions to maintain the permeability barrier at the TCJ (Fristrom, 1982).

The SJs are formed by a large complex of proteins, and the essential core complex consists of NeurexinIV (NrxIV), Coracle (Cor), Neuroglian (Nrg), the Na+/K+ ATPase pump (alpha and beta subunit) and Contactin, all of which are necessary for the formation and function of the SJs (Fehon et al., 1994; Baumgartner et al., 1996; Genova and Fehon, 2003; Paul et al., 2003). Mutations in the core SJ proteins result in loss of the septae and a disruption of the paracellular permeability barrier. This can be illustrated by the diffusion of small molecules across mutant epithelia (Lamb et al., 1998; Genova and Fehon, 2003) or into the nervous system (Auld et al., 1995; Bainton et al., 2005). The core components of the SJ complex are co-dependent for both complex formation and localization to the SJ (Bieber et al., 1989; Baumgartner et al., 1996;
Ward et al., 1998; Genova and Fehon, 2003). Beyond their role in creating permeability barriers, SJs are also necessary for the survival of columnar epithelial cells. For example, somatic clones for null mutations of Cor, NrxIV and Nrg are all cell lethal in the wing imaginal disc (Ward et al., 1998; Genova and Fehon, 2003), and it has yet to be determined why absence of the SJs causes lethality.

A cellular process that could be affected by SJ disruption is cell division. During cell division, the nucleus transitions from the basal side of the cell to the level of the SJ domain and remains there throughout mitosis (Gibson et al., 2006). Chapter two illustrates that cell division is localized exclusively within the SJ domain and organized in concert with SJ components, and Chapter 3 demonstrates that the SJs maintain the plane of cell division through localization of TCJ protein Gliotactin (Gli). Due to the potential involvement of the SJs in cell division illustrated in Chapters two and three, Chapter four investigates whether the SJs are necessary for mitosis and, in particular, cytokinesis.

All four stages of cytokinesis (formation of the cleavage furrow, assembly of the contractile ring, furrow ingression plus midbody formation and abscission) occur within the SJ domain, and are necessary in order for the progression of cytokinesis. A critical step in cytokinesis is the assembly of the contractile ring and the formation of the ingression furrow. For example, Anillin, a scaffolding protein recruited to the contractile ring assembly site, is essential for the organization of the contractile ring, ingression furrow stability and midbody formation (Somma et al., 2002; Echard et al., 2004; Straight et al., 2005; Zhao and Fang, 2005; D'Avino et al., 2008). A second contractile ring associating protein, Zipper, is necessary for initial contractions of the ingression furrow (Mabuchi and Okuno, 1977; De Lozanne and Spudich, 1987; Knecht
and Loomis, 1987; Straight et al., 2003), and proper assembly of the contractile ring is necessary for formation of the midbody (Eggert et al., 2006).

This chapter looks at how these four stages interact with the SJs, and asks whether a disruption in the SJ core protein NrxIV leads to defects in mitosis and cytokinesis. In particular, this chapter looks at the relationship between the SJs and the contractile ring. The formation of the cleavage and ingression furrows were assayed using the SJ markers, Dlg, Nrg, Gli and Fas III, the contractile ring was assayed using Anillin and Zipper, and the midbody was assayed using the microtubule-associating protein Jupiter (Karpova et al., 2006).

Downregulation of the SJs in the columnar epithelia of the wing imaginal disc leads to disruption of the ingression furrow and mislocalization of the contractile ring, and ultimately results in delamination and apoptosis of the mutant cells. This demonstrates an unexpected requirement for a permeability junction in cell division, and suggests that they are essential for cytokinesis. As these epithelial cells exhibit an architectural complexity shared by many vertebrate epithelia, the implications are that the mechanism of cytokinesis presented here may be conserved in vertebrate epithelia, involving the tight junctions and tricellular tight junction. It also suggests that there may be similar requirements for other vertebrate and invertebrate polarized epithelia for junctions outside of their classic adhesion and barrier roles.
Materials and Methods

Fly Strains

Stocks referred to as wildtype are w1118 or the GFP fusion protein lines outlined below. To
down-regulate the SJ core proteins in imaginal wing discs we used the following UAS RNAi
lines from the Vienna stock centre (Dietzl et al., 2007): UAS-cora RNAi (VDRC 9787 & 9788),
UAS-Nrg RNAi (VDRC 6688 & 27201), UAS-NrxIV RNAi (VDRC 8353 & 9039) and UAS-
Pebble RNAi (VDRC 35349 & 35350). We used the apterous-GAL4 (Bl: 106798) insertion line
from the Bloomington stock center. UAS-Dicer 2 (Dietzl et al., 2007) was co-expressed with
RNAi lines to increase RNAi efficiency and was obtained from the Bloomington stock center
(Bl: 24664). For wildtype analysis of cell division, we used GFP fusion proteins from the
Bloomington stock center or the Fly Trap Project, Dlg-GFP (CC01936), Zip-GFP (CC01626)
(Buszczak et al., 2007) and Jupiter-GFP (G00147), Nrg-GFP (G00305), (Morin et al., 2001).

Immunofluorescence

Immunolabeling of third instar imaginal wing discs was carried out as described previously
(Schulte et al., 2006). All images were generated on a Deltavision Restoration microscope
(Applied Precision). Data was collected using a 60X(1.4 NA) oil immersion lens using a
CoolSnap HQ digital camera. Data from all wavelengths was collected for each 0.2 um optical
section before the next section was collected. SoftWorx (Applied Precision) software was used
for deconvolution of 8-10 iterations using a point spread function calculated with 0.2 um beads
conjugated with Alexa Fluor 568, 488 and 647 (Molecular Probes) mounted in Vectashield.
Images were then exported to Photoshop CS4 for generation of figures.
Primary antibodies used in study were: mouse anti-Dlg 4F3 at 1:300 (Parnas et al., 2001), rat anti-DE-cadherin at 1:50 (Oda et al., 1994), mouse anti-Coracle (9C and C615-16B cocktail) at 1:500 each (Fehon et al., 1994), mouse anti-Gliotactin 1F6.3 at 1:100 (Auld et al., 1995) or rabbit anti-Gliotactin at 1:300 (Venema et al., 2004), rabbit anti-Anillin 1:600 (Goldbach et al., 2010), mouse anti-\(\gamma\)-tubulin 1:600 (Abcam), mouse anti-acetylated Tubulin 1:600 (Abcam), mouse anti-\(\alpha\)-tubulin 1:600 (Sigma), rabbit anti-phospho-Histone 3 1:600 (Abcam), mouse anti-Peanut 1:100 (Neufeld and Rubin, 1994), mouse anti-Fasciclin III 1:100 (Patel et al., 1987). All the following secondary antibodies were used at 1:200 dilution: goat anti-mouse, anti-rabbit or anti-rat conjugated to one of Alexa Fluor 488, Alexa Flour 568 and Alexa Fluor 647 or Cy5, (Molecular Probes). Nuclei were detected with DAPI at 1:1000.

**Ectopic expression of RNAi and assaying mitotic stages**

Crosses of individual RNAi lines were crossed to the apterous-GAL4 driver at progeny raised at 25\(^\circ\)C or 29 \(^\circ\)C. Imaginal discs were isolated from wandering 3\(^{rd}\) instar larvae and fixed following standard protocols (Schulte et al., 2006). Antibody stains were repeated a minimum of twice and 10 imaginal discs were imaged for each experiment to control for consistency. Discs that were damaged or otherwise varied from other wildtype samples were excluded.

For assaying the stage of mitosis, the areas assayed were exclusively in the wing pouch and images were collected using a 1024X1024 pixel area with a 60X objective (122X122 microns). Images were collected across the apterous dorsal/ventral border and thus for each region assayed, one half was composed of wild-type cells and the other half was apterous-GAL4 expressing cells. Each stage of mitosis was assayed as follows: metaphase and anaphase with DAPI, \(\gamma\)-
tubulin, phospho-Histone3 immunofluorescence; telophase A-C with Dlg, γ-tubulin, DAPI immunofluorescence.
Results

Organizing the ingression furrow and cytokinesis in columnar epithelia

The first goal of this chapter was to characterize the detailed changes that occur in the ingression furrow with respect to the SJs as the cells transition through telophase to abscission during cell division. At the onset of mitosis, the SJs widen in diameter to accommodate the nucleus (Chapter 2; (Gibson et al., 2006)). The next stage with observable changes in the SJ domain is at the start of telophase, when the ingression furrow forms. This is seen as an indentation on each side of the cell visible in the SJ domain (Figure 4.1A-C; Figure 4.2A).

During telophase, the centrosomes undergo an apical migration from the plane of cell division to reposition at the top (apical side) of the cell (Chapter 2). The progression of the centrosomes, in concert with SJ labeling, can be tracked through telophase A, B and C (Chapter 2). Using this staging approach, cells were immunolabeled for the centrosomes ($\gamma$-tubulin), the SJs (Neuroglian-GFP (Nrg-GFP)), and the TCJ (Gliotactin (Gli)). Cells were examined over the depth of the ingression furrow and single z stacks are shown over approximately 0.2 µm intervals. Three images are shown for each cell starting at the apical side of the ingression furrow (Figure 4.1A, E, I) and progressing through the center of the SJ domain (Figure 4.1B, F, J) to the basal reach of the ingression furrow (Figure 4.1C, G, K).

At the end of telophase A (Figure 4.1A-D), the SJ ingression furrow (Figure 4.1B, arrowhead) has progressed towards the center of the cell and reaches through the entire SJ domain (Figure 4.1A-B, arrowheads). At the basal region of the SJs, the ingression furrow transitions into a bicellular membrane marked by SJ proteins. This corresponds to the boundary between the SJ and the top of the descending cleavage furrow. (Figure 4.1C, arrowhead). At this stage, Gliotactin (Gli) is not associated with the ingression furrow (Figure 4.1B, arrowhead).
During telophase B, the ingression furrow continues to cinch towards the center but still remains open at the apical limit of the SJs (Figure 4.1E-H). Gliotactin marks the TCJs from the neighbouring tricellular corners associated with the apical side of the ingression furrow (Figure 4.1E, arrowheads), but is absent from the ingression furrow itself (Figure 4.1F-G, arrowheads). At telophase C, the apical sides of the ingression furrow meet to form a new apical septate junction (Figure 4.4I, arrows). This marks the transition of the ingression furrow from a furrow to a closed, tunnel-like structure contained within the SJ domain. The tunnel formed at telophase C is referred to from here on in as the SJ tunnel.

The transmembrane protein Gliotactin (Gli) is normally found concentrated at the TCJ in non-dividing epithelial cells. In telophase C, Gli is found on either side of the SJ tunnel at the new bicellular membrane corners (Figure 4.1I, arrowheads). At the level of the SJ tunnel itself, the ingression furrow is compressed together with projections from the two neighbouring cells still comprising part of the SJ tunnel. Although it appears as if Gliotactin marks four cornered junctions, this does not represent a four cell junction as there are technically only three cells present; the cell undergoing cytokinesis and the two neighboring cells. At the level of the SJ tunnel, Gli spreads along and lines the compressed ingression furrow (Figure 4.1J, arrowhead). However, Gli is absent from the basal side of the SJ tunnel (Figure 4.1K, arrowheads).

The formation of the new bicellular SJ and TCJs correlates with the consolidation of the SJ tunnel, suggesting that formation of the SJs, formation of new TCJs, and abscission are linked.

**The SJs form a tunnel during telophase**

The next step was to examine how the SJs interacted with the contractile ring and midbody during cytokinesis, as proper assembly of the contractile ring is necessary for formation of the
midbody and both structures are essential for cytokinesis (Egbert et al., 2006). Immunolabeling for Anillin was used to follow localization and assembly of the contractile ring (Field et al., 2005; Straight et al., 2005) and the microtubule-associating protein Jupiter, endogenously tagged with GFP, was used to mark the midbody (Karpova et al., 2006).

During telophase A, the contractile ring assembles between the two opposing sides of the ingression furrow and appears in z slices as a single band (Figure 4.2A, arrow). The midbody forms in the space between the two sides of the ingression furrow (Figure 4.2A, arrowhead). By telophase B, the single contractile ring seen in telophase A has split into two separate rings (Figure 4.2C, arrows). The ingression furrow takes on a more rigid structure (Figure 4.2B, arrowheads), and each of the rings is placed at the ends of the more structured ingression furrow (Figure 4.2C, arrows). By telophase C, the two bands of the contractile ring begin to dissipate (Figure 4.2E, arrows). The ingression furrow has transitioned into the SJ tunnel and the midbody is tightly packed inside, with the ends still protruding into each daughter cell (Figure 4.2E, arrowhead, asterisk). The final stage of cytokinesis, abscission, where the midbody is cleaved and the membrane completely sealed at the SJs, was not assayed as there is currently no marker available. However, at late telophase C, just before abscission, only remnants of the Anillin labeled contractile rings remain (Figure 4.2G, arrow), and the midbody still spans both cells (Figure 4.2G, asterisks). This suggests that in Drosophila epithelial cells, the contractile ring dissociates before abscission, and each daughter cell inherits a component of the midbody.

Whether the SJ tunnel formed at telophase C represented a true tunnel was assayed. A side projection was generated over the presumptive SJ tunnel in a telophase C cell immunolabeled for the SJs (Dlg) and the midbody (Jupiter-GFP) (Figure 4.3A-B). The SJs formed above and below the midbody that occupies the SJ tunnel (Figure 4.3B, arrowhead). These observations show that
between telophase B and C, the ingression furrow transitions into a tunnel that encases the midbody and is localized within the SJ domain.

These observations suggest that the contractile ring and midbody, two key components associated with the progression of cytokinesis, are strongly tied to the SJs, and that this localization may represent a functional relationship or role for the SJs in cytokinesis.

The Contractile ring markers Zipper and Anillin highlight distinct sub-compartments

The contractile ring localizes to the SJ tunnel throughout cytokinesis in columnar epithelia and appears to form two distinct rings. This is in contrast to the pattern shown in less architecturally complex cell types, where the contractile ring is shown as a single structure between two cells that overlaps with the midbody (Field et al., 2005; Straight et al., 2005). To look at the contractile ring in greater detail, cells were immunolabeled with Anillin and Zipper, two contractile ring markers that interact physically and are necessary for cytokinesis (Field and Alberts, 1995; Field et al., 2005; Eggert et al., 2006). Zipper and Anillin did not colocalize at the SJ tunnel past telophase A. Zipper was tightly associated with the ingression furrow (Figure 4.4C, arrowhead), whereas Anillin forms two rings and marks four points of the septate junctions tunnel (Figure 4.4C arrow) perpendicular to the ingression furrow and Zipper. Immunolabeling for Gli was used to confirm the specific sub-stage of telophase and to mark the SJ domain. At telophase C, Gli highlights the compressing ingression furrow and is observed flanking the SJ tunnel, which appears to bisect the contractile ring (Figure 4.4A, arrowheads). At this stage Gli localizes to the tip of the ingression furrow adjacent to Zipper-GFP and between the two Anillin labeled rings (Figure 4.4D, arrowhead).
A late telophase C cell is also shown prior to/or in the process of abscision (Figure 4.4E-F). By this stage, the Anillin immunolabeling is absent (Figure 4.4E, arrow) and the Zipper associated with the ingression furrow has formed a discrete ring (Figure 4.4E-F, arrowheads). Immunolabeling with Dlg highlights what remains of the SJ tunnel (Figure 4.4E). The localization of the Zipper ring adjacent to the remainder of the SJ tunnel suggests that this structure is inherited by one of the daughter cells (Figure 4.4E, arrowheads).

The transition of the ingression furrow to a ring as shown by Zipper GFP suggests that in columnar epithelial cells, abscission occurs when the SJ tunnel is excised, allowing the two tunnel membranes to anneal into one bicellular membrane between cells.

**Down-regulation of the SJ s leads to apoptosis**

In order to address whether loss of the SJ would lead to a disruption in cytokinesis, RNAi was used to down-regulate the SJ components. RNAi to the SJ core proteins was used instead of null mutants, due to the lethality and small clone size associated with SJ null clones (Genova and Fehon, 2003). Apterous-GAL4 was used to express RNAi in the dorsal half of the wing imaginal disc allowing mutant and wildtype tissue to be assayed side by side, eliminating variables such as antibody efficiency and variation between individual discs. The following studies concentrated on the core SJ protein NrxIV for RNAi analysis and used two independent RNAi constructs to control for off target effects. To access whether the SJs were mislocalized or disrupted in the columnar epithelia, discs were immunolabeled for the core SJ protein Coracle and the SJ associated protein Gli, both of which are mislocalized in NrxIV mutants (Genova and Fehon, 2003; Schulte et al., 2003).
Somatic clones of null mutants in the core SJ proteins results in a cell lethal phenotype (Genova and Fehon, 2003). Similarly, when NrxIV RNAi was expressed in wing epithelia, an accumulation of pyknotic nuclei was observed at the basal side of the epithelium (Figure 4.5I-J, arrowheads). This accumulation of pyknotic cells was also observed with Cora RNAi and Nrg RNAi (data not shown). Pyknotic nuclei in cells that had not yet delaminated from the membrane were also observed, as shown by non-junctional Dlg staining of the surrounding membrane (Figure 4.5A-C, arrowhead) and the presence of the normal interphase nuclei at this level of the epithelium. These observations coincide with previous results (Genova and Fehon, 2003) that suggested the SJs are necessary for cell survival in imaginal wing disc epithelia.

The cell lethal phenotype in somatic clones of mutations in the core SJ proteins was not due to a loss of cell polarity or the adherens junctions (Genova and Fehon, 2003). That the adherens junctions and apical/basal polarity were also unaffected in when core SJ proteins are knocked down with RNAi, was also confirmed (data not shown).

To address whether apoptosis could be linked to a failure to complete cytokinesis, cells were assayed for an association of pyknotic nuclei with cell division structures, such as the centrosomes and contractile ring. Pyknotic nuclei in NrxIV RNAi expressing cells showed an accumulation of the centrosome marker $\gamma$-tubulin (Figure 4.5I, arrowhead) and the septin protein Peanut, a contractile ring marker (Neufeld and Rubin, 1994) (Figure 4.5I, arrowheads). The localization of a contractile ring and centrosome marker with pyknotic nuclei in NrxIV RNAi mutant cells suggests that loss of the SJs leads to apoptosis during cytokinesis. It also suggests that cells mutant for SJs undergo cell death during telophase after the contractile ring has assembled.
Whether the nuclear envelope reformed in cells that were undergoing apoptosis was assayed to further pinpoint which stage of mitosis is disrupted. As the nuclear envelope reforms at the start of telophase, this serves as a staging point to determine whether mutant cells proceed past anaphase (Prokopenko et al., 1999). Cells were immunolabeled for Lamin C, a nuclear envelope marker, and mitotic nuclei were detected in the plane of cell division that were Lamin C positive (Figure 4.5E, H, arrowheads, inset). Pyknotic nuclei that were Lamin C positive were also detected (Figure 4.5G, arrowheads, inset), again indicating cells undergo apoptosis during telophase.

Multinucleate cells are a phenotype classically associated with failure to complete cytokinesis. For instance, Drosophila S2 cells lacking the contractile ring proteins Peanut and Anillin fail to complete cytokinesis and become multinucleate (Neufeld and Rubin, 1994; Shandala et al., 2004; Field et al., 2005; Straight et al., 2005). However, the only multinucleate cells observed in NrxIV RNAi epithelial cells had pyknotic nuclei (Figure 4.5E-G, arrowheads). Instances were also observed where cells appeared to go through cell division with extra nuclear material (Figure 4.5D). While this is a rare event in NrxIV RNAi treated discs, this phenotype was also observed in imaginal discs expressing Coracle and Neuroglian RNAi (data not shown).

The presence of multinucleate, apoptotic cells in NrxIV RNAi expressing discs raised the question of whether apoptosis was the default state for failure to complete cytokinesis in columnar epithelia. To address this, RNAi against Pebble, a Rho-GEF necessary for cytokinesis (Lehner, 1992), was expressed in columnar epithelia using the apterous-GAL4 driver, and the same pattern of pyknotic nuclei as seen with disruption of the SJs was observed, without evidence of surviving multinucleate cells (data not shown). Multinucleate cells were also not readily observed in the columnar epithelia of the imaginal disc in Citron kinase mutants, a
protein kinase required for mitotic spindle assembly and chromosome segregation (Shandala et al., 2004). These results suggest that failure to complete cytokinesis in columnar epithelia leads to cell death, and supports the idea that the SJs are necessary for the completion of cytokinesis.

**The SJs are necessary for the formation of the ingression furrow**

These results suggested that the NrxIV mutant cells failed to complete mitosis and that the critical stage might be telophase. Therefore, NrxIV RNAi cells were assayed to determine whether there was a discrepancy between numbers of cells at telophase in NrxIV RNAi versus wildtype cells. Cells were also assayed for proper spindle alignment to confirm that a block in mitosis was not due to an earlier defect. Immunolabeling for α-tubulin between metaphase and anaphase showed that the spindle positioning was normal in NrxIV RNAi expressing cells (Figure 4.6A-B, arrowheads). A quantification of cells in metaphase or telophase A indicated there was no difference between the average numbers of cells in equivalent areas of the wing imaginal disc on the mutant versus the wild-type side (metaphase: 2.2 wild-type cells versus 2.1 NrxIV RNAi cells, n=10 discs) (telophase A: 1.6 wild-type versus 1.1 NrxIV RNAi, n=10 discs). However, in NrxIV RNAi discs, cells that had progressed past telophase A were unidentifiable, due to the absence of the structured ingression furrow and SJ tunnel associated with telophase B-C (Figure 4.6C-D). There was no evidence of these stages on the mutant side, compared to an average of 1.4 cells in telophase B-C on the wild-type side (n=10 discs).

This was confirmed in a separate analysis using the centrosome marker γ-tubulin. In NrxIV RNAi cells, γ-tubulin immunolabeling never transitioned apically, a process that normally occurs by telophase C. For instance, in wildtype cells, we observed this repositioning at an average of 1.2 γ-tubulin pairs per area assayed (n=10 discs).
In the NrxIV RNAi treated cells, Dlg appears sparse and discontinuous at the region of the ingression furrow when compared to wildtype cells (Figure 4.6C-D). This becomes more apparent when cells are imaged at the plane of cell division (Figure 4.6D). However, the altered Dlg pattern was not seen in surrounding interphase cells (Figure 4.6D, asterisks). The discontinuous pattern of Dlg seen at the ingression furrow in NrxIV RNAi cells (Figure 4.6C-D) suggested there was a disruption in the membrane or a disruption in Dlg at the SJs. In order to determine whether the sparse Dlg staining was due to membrane or SJ disruption, a second SJ marker, Fasciclin III (FasIII), was used. FasIII is a SJ associated protein that is not mislocalized in an NrxIV mutant background (Genova et al., 2003), so would show the membrane at the ingression furrow in NrxIV mutant cells. Although FasIII highlights the inability of NrxIV RNAi cells to advance past the early formation of an ingression furrow (ie: telophase A) (Figure 4.6E-F, arrowheads), the membrane in both cells is clearly intact. This demonstrates that disruption of the SJs does have an effect on the localization of Dlg to the membrane at the ingression furrow and SJ tunnel, but not in surrounding interphase cells.

Overall, these results indicate that the defects in the NrxIV mutant cells are due to a failure to complete cytokinesis. The next question to address was whether the failure to complete cytokinesis was due to a malformation of the ingression furrow, or if there might also be an effect on the positioning and formation of the contractile ring.

**The SJs are necessary to maintain the contractile ring at the ingression furrow**

If the SJs help to localize or maintain the contractile ring at the ingression furrow, then in a NrxIV RNAi dividing cell, there should be an absence or mislocalization of the contractile ring markers. Anillin is localized normally to the ingression furrow at the start of telophase (Figure
4.6E-F, arrowheads; Figure 4.7A-E, arrowheads) and stills shows a normal localization pattern by telophase B (Figure 4.7F, arrowheads), suggesting that initial recruitment of Anillin and assembly of the contractile ring is normal in the absence of the SJ domain. The contractile ring was often observed asymmetrically localized to one side of the furrow in late telophase B-telophase C cells (Figure 4.7A-D), indicating a later effect on cytokinesis, as this is not seen in wildtype cells (Figure 4.7B, D, arrowheads).

To better evaluate the association of the contractile ring marker Anillin with the membrane, NrxIV RNAi expressing cells were immunolabeled with FasIII. Although the contractile ring showed the same asymmetric distribution to one side of the ingression furrow, as in Figure 4.7C-D during telophase B-C cells (Figure 4.7F-G), Anillin was continuously associated with the membrane (Figure 4.7E-G, arrowheads). These results suggest that the inability to form and stabilize a proper ingression furrow stems from a disruption in the association between the contractile ring and the SJs.

**Co-expression of P35 in cells ectopically expressing SJ RNAi blocks delamination**

If apoptosis was blocked in NrxIV RNAi cells would they delaminate and/or form multinucleate cells? To block apoptosis, p35 (a baculoviral inhibitor of apoptosis), was expressed concurrently with NrxIV RNAi. In the absence of apoptosis, cells did not delaminate basally, but also did not form multinucleate cells, suggesting that delamination is a side effect of apoptosis and not necessarily due to a disruption of the SJ domain. The lack of multinucleate cells is consistent with a classic class 2 cytokinesis mutant (Straight and Field. 2000; Straight et al. 2005), but may also be a result of disrupting cytokinesis in architecturally complex columnar epithelia.
Discussion

The septate junctions and cytokinesis

In contrast to Drosophila S2 cells and embryonic epithelia, columnar epithelial cells exhibit a high degree of architectural complexity. This raises the question of how much of the current model of cell division determined in these simpler cell types is applicable to architecturally complex cells, and what modifications have been made to accommodate for these structural differences.

In columnar epithelia, cell division is localized to the SJ domain. At the onset of telophase, the ingression furrow, contractile ring and midbody, form at the SJs and remain there until the daughter cells have separated. During cytokinesis, the SJs form a tunnel where the midbody and contractile rings localize. Chapter four demonstrates that the association of the SJs with these key components serves a functional purpose during cytokinesis. Downregulation of the SJs affects the formation of this tunnel and leads to a disruption of the ingression furrow, mislocalization of the contractile ring, and apoptosis.

The link between the septate junctions and the contractile ring

In Nrx IV knockdown no mitotic cells were observed beyond telophase A, suggesting that the SJs fail to form at the new membrane and that the ingression furrow does not transition into the SJ tunnel. The ingression furrow does not appear to invaginate properly and the contractile ring is no longer secured equidistant to the opposing membranes. It could be that in absence of the SJs, the membrane is unlinked from or unable to respond properly to the contraction of the actomyosin contractile ring. The fact that the contractile ring still assembles and contracts in absence of the SJs supports this hypothesis.
In addition, Dlg was discontinuous at the ingression furrow, suggesting that the SJs are necessary for maintaining Dlg at the membrane in dividing cells. FasIII labeling verified that the membrane itself is intact, indicating that the SJs are necessary for the formation of the ingression furrow but not membrane integrity. This result, paired with the failure of the contractile ring to be aligned equatorially between opposing membranes, suggests the SJs are necessary for maintaining the ingression furrow.

Potential links between the SJs and the contractile ring are the SJ associated proteins Dlg and lethal giant larvae (Lgl). During asymmetric cell division in Drosophila SOP, Dlg is involved in establishing the plane of cell division and cleavage furrow through an association with Kinesin heavy chain 73 (Khc73), a plus-end microtubule motor protein that binds to the GUK domain of Dlg (Betschinger and Knoblich, 2004; Ahringer, 2005; Siegrist and Doe, 2005). This interaction is conserved between hDlg and the human homologue of Khc73, Gakin/MAPDA (Yamada et al., 2007). In HELA cells, mutations in hDlg lead to failure of cytokinesis and multinucleate cells (Unno et al., 2008), and hDlg and Gakin are necessary for maintaining furrow stability and architecture during late cytokinesis (Unno et al., 2008). Due to the conserved interaction, it is possible that Dlg and Khc 73 are also necessary for maintaining furrow integrity in columnar epithelia, and that the SJs are necessary to maintain Dlg continuously at the membrane. If this is the case, then downregulation of Dlg in the wing imaginal disc should lead to a similar mislocalization of the contractile ring as seen here.

The other possible link between the septate junctions and the contractile ring is lethal giant larvae (Lgl). Lgl is a polarity protein that interacts in a complex with Dlg and Scribble, which are localized to the SJ domain (Bilder et al., 2000). During asymmetric cell division in neuroblasts cells, Lgl establishes the plane of cell division (Betschinger and Knoblich, 2004) by
inhibiting the association of Zipper with the cell cortex (Strand et al., 1994; Kalmes et al., 1996; Betschinger and Knoblich, 2004). Since Lgl interacts with Dlg and Scrib, in columnar epithelia (Bilder et al., 2000) it is possible that the interaction between Lgl and Zipper is the link between the SJs and the contractile ring. In this scenario, mislocalization of either Dlg and/or the SJs would lead to a disruption of Lgl, breaking the link with Zipper.

**SJ mutants are not classic cytokinesis mutants**

In S2 cells, mutations in the cytokinetic ring proteins Anillin, Peanut and Zipper, lead to an accumulation of multinucleate cells due to a failure to complete cytokinesis (Neufeld and Rubin, 1994; Shandala et al., 2004). Unlike these classical cell division mutants, failure to complete cytokinesis in NrxIV RNAi expressing cells leads to delamination followed by apoptosis. Usually where the contractile ring is destabilized or absent, cells are either unable to initiate ingression or proceed to a point where the midbody and abscission are un-absolved and the contractile ring “relaxes”. These are referred to as class one and class two cytokinesis mutants respectively. In these instances the cell membrane regresses to the starting point leaving binucleate cells (Neufeld and Rubin, 1994; Somma et al., 2002; Field et al., 2005). Although binucleate cells with pyknotic nuclei were observed, there was no evidence of living binucleate cells.

Apoptosis is not a phenotype normally associated with cytokinesis mutants, and this is likely due to the predominance of cell division studies in simpler cell types. A case in point is the down-regulation of Citron kinase, which generates binucleate cells in S2 cell culture (Naim et al., 2004). In contrast, the absence of Citron kinase in the imaginal wing disc leads to apoptosis (Shandala et al., 2004). This suggests that living multinucleate cells only occur in some cell
types, and presumably failure to complete cytokinesis in architecturally complex epithelia results in apoptosis. The ectopic expression of pebble RNAi in columnar epithelium, which resulted in apoptosis, further supports this hypothesis, and would explain why the phenotype has not been reported extensively.

One reason for a default apoptosis could be due to a checkpoint in architecturally complex cells that does not allow for relaxing of the ingression furrow or disassembly of the contractile ring past a certain point. A transition past this check point would result in an irreversible furrow that cannot be resolved, and would necessitate apoptosis. A third possibility is that it is simply not feasible to reverse new membrane formation in telophase cells of the imaginal wing disc. During cytokinesis, columnar epithelia must synthesize a new membrane between the two new daughter cells. Considering that this synthesis is initiated at the cleavage furrow by metaphase, by telophase C the new membrane of ~30 microns is almost finished. By this stage, even if the contractile ring were to collapse, it is likely that cytokinesis has proceeded to far to be effectively reversed.

**Zipper as a marker for abscission**

The presence of circular structures in late telophase outlined by Zipper-GFP were intriguing and reminiscent of the intercellular bridge marker, Cindr, in the imaginal wing disc (Haglund et al., 2010). It is an interesting observation as Zipper is an essential component of the contractile ring, but this structure appears to localize independent of the Anillin recruitment factor or the contractile ring. It is possible that the ring formed by Zipper represents an excision model for abscission, where the membrane is negotiated by excising the ingression furrow. Whether disruption of the SJs will affect this ring structure remains to be seen.
Conclusion

This chapter illustrates that the SJs have been recruited into a unique role in architecturally complex wing epithelium for cytokinesis that is unexpected and likely separate from their role in maintaining permeability barriers. It also illustrates a solution to an interesting problem; how to organize and execute cell division in a cell that must maintain its junctions while dividing. The involvement of the SJs in cytokinesis in the polarized cells of the Drosophila imaginal wing disc introduces the idea that junctions in other systems could also perform similar roles. For example, in MDCK cells the formation of a tunnel like structure at the level of the tight junctions has been observed (Reinsch and Karsenti, 1994). This already points towards the permeability barrier as a location and structural component necessary for cytokinesis in epithelia cells in general. Although there is the mechanistic question of how the bicellular membrane is negotiated at a molecular level, the involvement of septate junctions and other permeability barriers opens a starting point for further understanding this cellular process.
Figures

Figure 4.1 telophase and abscission occur at the septate junctions in columnar epithelia

Wildtype cells from 3rd instar imaginal wing discs, from late telophase A to telophase C. Cells are immunolabeled for the septate junctions (Nrg-GFP, red), the tricellular corner (Gli, green) and the centrosomes (γ-tub, blue).

A-D) A late telophase A cell imaged over 12 microns, from the top of the septate junctions (A) to the basal side (C). During telophase A the septate junction ingression furrow is open at the apical side (A, arrowheads). The descending cleavage furrow/new bicellular membrane can be seen at the basal region of the septate junctions (C, arrowheads). A side projection (D) stages the cell as telophase A from the position of the centrosomes within the SJ domain. Arrowheads indicate the descending cleavage furrow.

E-H) telophase B cell imaged over 7 microns. The ingression furrow has started to pinch in and Gliotactin can be found on either side (E, arrowheads). The cells are staged as telophase B by the progression of the centrosomes (H, arrows). Arrowheads indicate the descending cleavage furrow.

I-L) telophase C cell imaged over 9 microns. The apical side of the ingression furrow has formed a new bicellular membrane (I, arrows) with two new tricellular junctions (I, arrowheads, Gli) on either side. Gli can now be found lining the ingression furrow (J, arrowheads). Cells are staged as telophase C by the progression of one of the centrosomes above the SJ domain (L, arrow). Arrowheads indicate new bicellular membrane.
Figure 4.2 The midbody and contractile ring are localized to the septate junction tunnel during telophase

Cells at each stage of telophase (A-F) and late telophase C (G) shown immunolabeled with a contractile ring marker, Anillin (red), a septate junction marker, Dlg (green) and a midbody marker, Jupiter-GFP (blue). (B,D,F) Side projections of cells shown in A,C,E re-sectioned across the dashed line.

A) By telophase A, the contractile ring has started to form around the midbody (arrow). At this stage the ingression furrow at the level of the SJs can be seen to constrict on either side of the midbody (arrowhead indicates the space midbody occupies). B) The single contractile ring (arrow) localizes to the center of the midbody (arrowhead). Note that this structure is representative of a ring in cross section.

C-F) By telophase B, the contractile ring has split into two distinct parts (C, arrows). Each half of the contractile ring is placed at the end of a tunnel formed by the SJs and the ingression furrow (C, arrowhead). This localization continues through to telophase C (E, arrows, arrowheads). The two contractile rings (D, arrows) are localized to the midbody (D, arrowhead) that has condensed at the center of the forming SJ tunnel. At telophase C the ends of the midbody still protrude through the sides of both daughter cells (E, stars), and the contractile ring is still localized around the midbody (F, arrows).

G) At late telophase C, prior to abscission, the midbody, contractile ring and ingression furrow at the SJs still remain. The remnants of the contractile ring can be seen faintly at the end of the SJ tunnel (arrow). The midbody is still present and still protrudes into both daughter cells (stars). The SJ tunnel formed by the ingression furrow can still be seen faintly around the midbody (arrowhead).
Scale bars represent 7.5 microns.

Scale bars in A-C represent 5 microns, D represent 5 microns. Each panel represents a single Z slice of 0.2 microns.
Figure 4.3 The septate junctions encase the midbody within a tunnel during telophase

A-B) A closer investigation of the SJ tunnel using Jupiter-GFP to mark the midbody and Dlg to mark the SJ. Panel B is a rotation of the square indicated in A (arrowhead). The SJ tunnel can be seen pinching around the midbody (arrow). When this small area is rotated, the SJ tunnel (arrowhead) can be seen on either side of the midbody (arrow).

The enface panel in A represents a single Z slice of 0.2 micron. Scale bars represent 2.5 microns.
Figure 4.4 Zipper-GFP and Anillin localize to different regions of the contractile ring during late cytokinesis in columnar epithelia

A-B) Columnar epithelia immunolabeled for the ingression furrow at the SJs (Gli, green) and the contractile ring (Anillin, red). Gli illustrates the ingression furrow flanking the contractile ring during telophase C (arrowheads). B is a z-slice taken 0.6 microns apical to A.

C) Telophase C cell immunolabeled for Anillin (red), Zipper-GFP (blue) and Dlg (green). Anillin marks four points of the septate junction tunnel (C, arrowhead), and Zip marks the ingression furrow (C, arrow). D) Telophase C cell immunolabeled for Gli (green), Anillin (red), and Zipper (blue). Gli is located behind the leading edge (Zipper) of the ingression furrow (D, arrowhead).

E) Late telophase C cell before abscission immunolabeled for Anillin (red), Dlg (green), and Zipper (magenta). Dlg indicates the remainder of the SJ tunnel (E, arrow). Anillin is absent, but Zipper still remains in a circular structure (E, arrowhead; F, arrowhead). F) Late telophase C cell immunolabeled for Gli (green) and Zipper (magenta). Gli indicates the new TCJ.
**Figure 4.5 Downregulation of Neurexin IV leads to delamination and apoptosis**

A) NrxIV RNAi cells imaged basal to the septate junctions in the plane of the interphase nuclei. Dlg immunolabeling (green) at this plane is non-junctional and labels the membrane. Arrowheads indicate multinucleate cell with pyknotic nuclei labeled with DAPI (blue).

B-D) NrxIV RNAI pyknotic, multinucleate cells. B) The inset cell indicated in A at ~10X magnification. Arrowhead indicates blue pyknotic nuclei. C) Another example of a multinucleate pyknotic cell (arrowhead). E) A multinucleate cell at the plane of cell division (z30) labeled with mitotic marker phospho-histone 3 (PH3, red). The cell outline (arrows) is indicated by Coracle (Cora, green), which has been digitally enhanced to compensate for the extensive down-regulation in the NrxIV RNAi background. PH3 indicates the cell is in anaphase. DNA marker, DAPI (blue) labels the nuclei. Arrowhead indicates pyknotic nuclei.

E-H) NrxIV RNAi cells have are positive for nuclear membrane marker Lamin. E) Plane of cell division, F) half way through the epithelium, G) Below the basal membrane. E) arrowhead indicates wildtype prophase cell (inset) at the plane of cell division. F) cells basal to the SJs. Arrowhead indicates mutant cells labeled with Lamin (inset). G) mutant cells that have delaminated from the membrane, are pyknotic and are labeled with Lamin. Arrowheads indicate cells that are shown in inset panel at ~5x magnification. H) A side projection through the Disc showed in E-G. Vertical arrow indicates border between wildtype and mutant cells (mutant right of arrow). Star indicates wildtype cell shown in E. Arrowhead indicates mutant cell labeled for Lamin below the wildtype plane of cell division. Arrow indicates delaminated pyknotic nuclei also labeled with Lamin.

I) A side projection across the dorsal/ventral boundary in an NrxIV RNAi expressing disc. Vertical arrow indicates wildtype mutant boundary (mutant tissue is right). On the NrxIV RNAi
side, Peanut (red, arrowhead) is associated with the delaminated pyknotic nuclei (blue, arrowheads).

J) A side projection across the dorsal/ventral boundary in an NrxIV RNAi expressing disc. Vertical arrow indicates wildtype mutant boundary. Gliotactin (red) mislocalization indicates mutant tissue (right of arrow). Centrosome marker $\gamma$-tub (green) is associated with the delaminated pyknotic nuclei (arrowhead).
Figure 4.6 Neurexin IV RNAi expressing cells divide but fail to form a proper ingression furrow

A-B) NrxIV RNAi expressing columnar epithelia at metaphase and anaphase. Phospho histone 3 (PH3, red) marks the nuclei and α-tubulin (α-tub, green) marks the spindle. The spindle forms properly in these cells (arrowheads).

C-D) NrxIV RNAi cells do not progress beyond telophase A-B. The SJ was visualized in two different planes using two SJ markers, Dlg (green) and Gliotactin (Gli, red).

C) At 3.2 microns below the plane of cell division, basal to the focal plane in D. The SJ markers, Dlg (green, arrowheads) and Gliotactin (red) highlight the formation of the ingression furrow.

D) At the plane of cell division, apical to the focal plane in B. The SJ marker Dlg (green) highlights the failure to form an ingression furrow. Note the spotty appearance of Dlg at the ingression furrow (arrowheads).

E-F) NrxIV RNAi cells at telophase A immunolabeled for the SJ protein FasIII (green), Anillin (red), and DAPI (blue). Cells form asymmetric ingression furrows (arrowheads). E) Anillin marks the emerging ingression furrow. The two constriciting sides do not do so in a way that will divide the cell evenly (E, arrowhead). Note the extra nuclear material (E, DAPI, arrow). F) Anillin localizes to the ingression furrow site, but only one side has contracted (F, arrowheads).

Scale bars represent 5 microns in panels B-C. All enface panels represent a single Z slice of 0.2 microns.
Figure 4.7 Loss of the septate junctions lead to a defect of ingression furrow maintenance around the contractile ring and a failure in cytokinesis

A-D) Expression of NrxIV RNAi using apterous-GAL4 immunolabeled for the contractile ring (Anillin, red), the septate junctions (Dlg, green) and the nuclei (DAPI, blue). NrxIV RNAi cells are at telophase B-C as indicated by the double Anillin ring (arrowhead). Dlg is disrupted at the ingression furrow as determined by Dlg immunolabeling (arrows). A wider view of an NrxIV RNAi cell is shown in A indicating that Dlg is not discontinuous and normally distributed in the surrounding interphase cells (arrow).

E-H) Expression of NrxIV RNAi using apterous-GAL4 immunolabeled for the contractile ring, (Anillin, red), the septate junctions (FasIII, green) and the nuclei (DAPI, blue). Cells are shown at telophase A (E), telophase B (F) and telophase C (G-H). The contractile ring doublets are mislocalized from the ingression furrow (G, H, arrowheads). The ingression furrow contracts unevenly (E, arrowheads), but progresses until telophase B (F, arrowheads).
V. General Discussion

Gli and the SJs are classically identified as components necessary for establishing and maintaining the permeability barrier across membranes. Outside this role, SJs maintain apical/basal polarity during embryogenesis (Laprise et al. 2009), and regulate size of the apical membrane (Wu and Beitel. 2004). In this thesis, a new role is demonstrated for Gli and the SJs during cell division, where Gli is necessary to maintain the plane of cell division and the SJs are necessary for cytokinesis and maintaining the structural integrity of the ingression furrow.

Organization of cell division in architecturally complex cells

Cell division was mapped out in columnar epithelia taking into consideration their architectural complexity. This step was necessary in order to characterize cell division phenotypes in SJ and Gli RNAi mutants. When the major structures and stages of mitosis and cytokinesis were correlated with the SJ domain, previously unidentified structures, such as the α-tubulin cap, midbody bridge, a centriole-like structure, and abscission ring were identified and described. These structures highlighted differences in the organization of cell division between architecturally complex cells and S2 cells. For example, the midbody and midbody bridge (tubulin associated with both ends of the midbody throughout cytokinesis) overlap in S2 cells and are indistinguishable as individual structures (reviewed in Eggert et al. 2006). One possible reason for this organization in columnar epithelia is that the midbody bridge acts as a structural tether to maintain the midbody at the SJs when the nuclei migrate basally. Another is that it serves as a tract for the apical migration of the centrosomes during telophase.

At the end of cytokinesis, a portion of the Drosophila midbody appears to be inherited by each daughter cell, and not secreted apically (Golsteyn et al., 1994; Hinchcliffe, 2005; Dubreuil
et al., 2007) or inherited asymmetrically by one daughter cells, as occurs in vertebrate cells and S2 cells (Mishima et al., 2002; Gromley et al., 2005; Straight et al., 2005; Goss and Toomre, 2008). It is possible the difference in midbody inheritance observed in columnar epithelia is due to the reversed positions of the permeability TJs and SJs. In vertebrate epithelia, the apical location of the tight junctions above the adherens junctions might make single inheritance or apical secretion the easiest means to resolve the midbody, whereas in Drosophila, since the SJs are localized basal to the adherens junctions, dividing or cleaving the midbody may be more efficient.

Although Drosophila epithelia, unlike vertebrate epithelia, do not have centrioles (Basto et al. 2006) an argument can be made for an analogous structure. In columnar epithelia, the centriole associating markers γ-tubulin and Polo kinase, relocalize to the SJs and back to the apical side of the cell during cell division (Chapter 2). This is similar to centriole migration in vertebrate epithelia, such as MDCK cells, where the centriole is recruited to the plane of division from the apical side of the cell during mitosis, and back to the apical side of the cell during telophase (Reinsch and Karsenti, 1994; Baye and Link, 2007; Schenk et al., 2009). A centriole-like microtubule organizing centre (MTOC) has already been found in tracheal cells that relocalizes after cell division to the apical domain (Brodu et al., 2010), further supporting the presence of this structure on columnar epithelia.

Vertebrate nuclear migration is in part thought to be a mechanism for the nucleus to meet up with the apically stored centriole (Reinsch and Karsenti, 1994), and this may also be true for Drosophila epithelia as well. In vertebrate epithelia, centrioles also form the base of the primary cilia. Since Drosophila epithelia do not form primary cilia (Basto et al. 2006) it is possible that a
How is the spindle targeted to the SJ domain?

In columnar epithelia, the centrosomes of mitotic cells localize to the TCJ, which suggested a role for the TCJ in organizing the spindle and plane of cell division. However, since the centrosomes and spindle still orient properly with respect to the cell cortex and associate with a TCJ in Gli and SJ RNAi expressing cells, it is likely that another component at the TCJ, besides Gli, acts to orientate the spindle. One candidate is Dlg, as Gli and Dlg are the only two known proteins to concentrate at the TCJ (Schulte et al., 2006). Little is known about how the spindle is positioned at the cortex, but one way this could be accomplished is through the heterotrimeric G-proteins (α, β and γ- subunits), which play an important role in spindle orientation during asymmetric cell division. Along with Bazooka/Par3, Par6 and Pins, the G-proteins mediate interactions between cortical polarity cues and the mitotic spindle (Wang et al., 1990; Roychowdhury et al., 1999). It is possible that the interaction between the G-proteins and Bazooka/Par3 is conserved in columnar epithelia, and that the Bazooka/Par 3 complex is responsible for organizing the spindle through an interaction with the G-proteins.

Although Gliotactin and the SJs are not necessary for the orientation of the spindle with respect to the cortex, they are necessary to maintain the plane of cell division. Columnar epithelia undergo nuclear migration during cell division; at the onset of mitosis the nucleus transitions to the SJ domain where it remains until cytokinesis, when the daughter nuclei transition back to the basal side of the cell. This process is reminiscent of interkinetic nuclear migration and translocation seen in vertebrate neuroepithelial cells and Drosophila.
photoreceptors (reviewed in Baye and Link. 2008), and raises the question of whether nuclear migration is a general theme for cells with both height and extensive junctional domains, and also raises the question of whether this migration is an essential component of cytokinesis.

When Gli was downregulated and/or mislocalized at the membrane through the expression of Gli RNAi or SJ RNAi, the plane of cell division was mislocalized. There are two ways that loss of Gli could be affecting the plane of cell division: a failure of the nuclei and centrosomes to be held at the SJ domain, or a failure of the nucleus to reach the correct location. However, the failure to detect mitotic Gli null clones or show apical mitotic nuclei, suggests that Gli is necessary for apical nuclear migration.

How does Gli affect the plane of cell division? A stabilized network of microtubules (Chapter 3) is concentrated at the tricellular corner directly below the SJs in columnar epithelia. The mislocalization of both α-tubulin and the microtubule motor protein Kinesin when Gli is mislocalized suggests Gli maintains this network. However, Gli could also be disrupting nuclear migration through a disruption of the microtubules and their associated motor proteins. Kinesin and Dynein are necessary for transporting the nucleus along the microtubules during interkinetic nuclear migration through their association with conserved nuclear envelope proteins, such as KASH and SUN (reviewed in Baye and Link. 2008). Kinesin is typically responsible for the nuclei to initiate apical migration, and Dynein is responsible for the nuclei to descend during cytokinesis, however, this depends on the orientation of the +/− ends of the microtubules in the cell, and is expected to be reversed in columnar epithelia (Mogensen et al., 1989; Fridolfsson and Starr, 2010). If Gli and the tricellular corner form an organizational site for the cytoskeleton, then mislocalization could be leading to a disruption in the localization of Dynein/Kinesin motor proteins.
Gli RNAi expressing discs also failed to reposition γ-tubulin at the apical side of the cell, and this may be another phenotype resulting from a disruption in the microtubule cytoskeleton and/or motor proteins. The motor protein Kinesin is involved in vertebrate centriole positioning and ciliogenesis through interaction with polarity proteins (Fan et al. 2004). If the interaction between Kinesin and the Drosophila centriole-like structure is conserved, than the mislocalization may also be conserved in Drosophila epithelia with the centrosome-like structure.

Future directions include down-regulating Kinesin and Dynein in columnar epithelia. If knockdown of these motor proteins show the same disruption in the plane of cell division and mislocalization of apical γ-tubulin seen with Gli RNAi, then this suggests downregulation of Gli is disrupting the plane of cell division and γ-tubulin mislocalization through changes in the microtubule network.

The SJs maintain furrow integrity during late cytokinesis

The SJs maintain the plane of cell division through the proper localization of Gli. However, NrxIV RNAi expressing cells also failed to maintain the ingression furrow integrity during late cytokinesis, which was independent of any effect on the localization of Gli. Dlg at the ingression furrow in these cells became discontinuous, suggesting that the SJs are necessary for maintaining Dlg at this membrane in dividing cells. This result, paired with the tendency of the contractile ring to localize to one of the late ingression furrow between opposing membranes, suggests the SJs are necessary for maintaining the ingression furrow.

How could downregulation of the SJs affect ingression furrow integrity? One candidate is Dlg, which has a role in assembly of the asymmetric cleavage plane, and maintains ingestion
furrow integrity in vertebrate epithelia (Betschinger and Knoblich, 2004; Unno et al., 2008). During asymmetric cell division in neuroepithelia and imaginal disc sensory organ precursors, Dlg regulates the orientation of the cleavage plane through Kinesin heavy chain 73 (Khc73) and Pins/G-proteins (Betschinger and Knoblich, 2004; Ahringer, 2005). Human Dlg (hDlg) binds and activates GAKIN ATPase activity through a GUK domain (Yamada et al., 2007), and in HEKA cells, hDlg and GAKIN maintain furrow stability and architecture during late cytokinesis (Unno et al., 2008). It is possible that Dlg and Khc73 also maintain furrow integrity in columnar epithelia, especially since the binding interaction is conserved. If the SJs are necessary to maintain Dlg at the ingression furrow, than downregulation of the SJs could destabilize Dlg and Khc73 at the furrow. In this scenario, downregulation of Dlg in the wing imaginal disc should also lead to a disrupted ingression furrow and mislocalization of the contractile ring, as seen in NrxIV RNAi expressing cells.

A major point that remains unclear is the link between the septate junctions and the contractile ring, and why the contractile ring is dissociated from the membrane in NrxIV expressing cells. Lgl is a polarity protein that interacts in a complex with Dlg and Scribble, two proteins that are concentrated at the septate junctions in imaginal discs (Bilder et al., 2000). At the start of telophase, Zipper initiates the ring contraction that forms the ingression furrow (Mabuchi and Okuno, 1977; De Lozanne and Spudich, 1987; Knecht and Loomis, 1987; Straight et al., 2003). Lgl helps establish the asymmetric plane of cell division in neuroblasts cells (Betschinger and Knoblich, 2004), partly through inhibiting the association of Zipper with the cell cortex (Strand et al., 1994; Kalmes et al., 1996; Betschinger and Knoblich, 2004). If Lgl localizes to the SJs in columnar epithelia, and the interaction with Zipper is conserved, than Lgl may be linking the contractile ring to the membrane. If NrxIV or Dlg maintain Lgl at the SJs,
then downregulation of NrxIV could disrupt this association, leading to the mislocalized contractile ring late in telophase.

There are two possible ways that Lgl could interact with Zipper at the contractile ring in columnar epithelia. The simplest model is that Lgl is necessary at the SJs to hold the contractile ring in place (Figure 5.1B). Disruption of the SJs would lead to mislocalization of Lgl and a dissociation of the membrane form the contractile ring as seen in Figure 4.7E-G. It is also possible that the inhibitory interaction between Lgl and Zipper is carried over into the columnar epithelia. In this instance, Lgl would need to be absent from the SJs at the ingression furrow to allow for localization and/or or activity of Zipper (Figure 5.1C).

Future directions include determining whether Zipper localizes properly to the ingression furrow in SJ RNAi expressing cells, and assaying both Lgl and myosin II for mislocalization at the ingression furrow in NrxIV and Dlg RNAi backgrounds. Another goal would be to identify and investigate more candidate proteins that could link the SJs to the contractile ring. One candidate from vertebrates is the TJ protein Cingulin, which acts as a crosslink between the membrane cytoskeleton and myosin in the contractile ring (Cordenonsi et al., 1999; Fanning et al., 1999; Citi et al., 2000). A Cingulin homologue or binding partner could also be assayed in the columnar epithelia to determine if it localizes to the SJs and ingestion furrow during cytokinesis, and whether it showed mislocalization in NrxIV RNAi expressing cells.

The subdivision of Zipper and Anillin at the contractile ring

It was surprising and unexpected that Zipper and Anillin showed such distinct localization patterns in the contractile ring and ingression furrow of columnar and peripodial epithelial cells. In S2 cells and embryonic epithelia, both contractile ring markers show pattern of co-localization
to the contractile ring. Their patterns are indistinguishable and do not indicate a sub-localization (Fields et al. 2005; Straight et al. 2005). There are two possible explanations for the contractile ring sub-domains demonstrated in columnar epithelia and peripodial cells. The first is that the sub-domains exist in all contractile rings, but in architecturally simple cells, they are compacted into a small space. The second explanation is that in architecturally complex cells, the contractile ring is subdivided for structural purposes, such as the rigid nature of the SJ tunnel. In S2 cells, Anillin interacts directly with Zipper, however, they are both targeted and localized independently to the contractile ring (Straight et al. 2005). This supports the idea that S2 cells also have contractile rings with sub-domains seen in columnar epithelia. Since Anillin and Zipper do not colocalize in telophase C, determining whether Anillin is still required for the proper contraction of Zipper during late cytokinesis in columnar epithelia is important as this would imply that the mechanics of the contractile ring change with increased architectural complexity.

**Apoptosis in Gliotactin and SJ RNAi is likely due to failure to complete cytokinesis**

There are two potential reasons Gli and SJ RNAi expressing cells undergo apoptosis; a failure to complete cytokinesis or disruption of an independent process. Although the Gliotactin and SJ RNAi expressing discs showed a correlation between disruption in cell division and apoptosis, it was not possible to exclude that interphase cells were also undergoing apoptosis, or that the two phenotypes were due to independent effects. Disruption of essential cytokinesis proteins Citron Kinase and Pebble cause apoptosis in the imaginal wing disc (Shandala et al., 2004; Gregory et al., 2007), not an accumulation of multinucleate cells as is classically seen in S2 cytokinesis mutants (Straight et al. 2005). This could be due to the nature of columnar epithelia and the
extensive membrane that has to be deposited between the two daughter cells. A failure in cytokinesis would require the disassembly of the membrane, and this might not be feasible at this late stage.

Although the ingression furrow is unaffected in Gli RNAi expressing discs, it is possible that mislocalization of the plane of cell division leads to a failure in a cytokinesis that could still triggers a default of delamination and apoptosis. That the contractile ring protein Anillin only assembled in Gli RNAi mutant cells that made it to the SJ domain, and was never seen associated with centrosomes below the SJ domain, supports this hypothesis as it suggests that essential components of the contractile ring are unable to assemble below the SJ domain. If this is the case, then Gli RNAi cells dividing below the SJ domain would be unable to undergo cytokinesis.

Analysis of multiple contractile ring markers in a Gli RNAi background, such as Zipper-GFP and Polo-GFP, will help to determine if the absence of Anillin in those cells that fall below the SJ domain applies to contractile ring proteins in general. However, an alternative argument is that disruption of the microtubule network in Gli RNAi cells leads to apoptosis through disrupting vesicle transport to the newly forming membrane or inducing apoptosis through a pathway independent of the mislocalized plane of cell division. Although a possibility, the second hypothesis is less likely, as disruption of the microtubules in the imaginal disc epithelia has not been associated with apoptosis. Disruption of the microtubules was seen in Dlg and Lgl mutant discs associated with the over growth and proliferation defects, but unaccompanied by apoptosis (Bryant et al. 1988; Woods and Bryant. 1989). Similarly, disruption of microtubules through ectopic expression of Spastin, a microtubule severing AAA ATPase, also did not lead to apoptosis in the imaginal discs (Chen et al., 2010).
Vesicle targeting and membrane formation

The deposition of new membrane through targeted vesicle insertion at the cleavage and ingression furrows is an essential component of cytokinesis independent of the contractile ring and furrow ingression (Bluemink and de Laat, 1973; Drechsel et al., 1997; Glotzer, 2001; Eggert et al., 2006). Although no phenotypes are reported in this thesis that affected membrane deposition, vesicles must be targeted to the SJ tunnel during cytokinesis.

Soluble NSF attachment protein (SNAP) receptors (SNAREs) are incorporated into budding transport vesicles through the secretory pathway (Fasshauer et al., 1998; Weber et al., 1998) and their main role during cytokinesis is to mediate vesicle fusion to the membrane. Functionally, the SNAREs can be divided into two categories; targeting SNAREs, (t-SNARE/Syntaxin) and vesicle-associated membrane protein (VAMP; v-SNARE) (Hong, 2005). How t-SNAREs and v-SNAREs are targeted to the SJs during cytokinesis is unknown, but three possibilities are through the centrosome/centriole-like structure, the SJ associated protein Lgl, and Anillin at the contractile ring.

In vertebrates, the centrosome protein, centriolin, localizes to the contractile ring during abscission and acts as a scaffold between the exocyst complex on targeted vesicles and the SNARE complex at the ingression furrow (Gromley et al., 2005; Eggert et al., 2006). It is possible that the centrosomes in columnar epithelia perform a similar role.

Although Drosophila Lgl has not been implicated in vesicle fusion, both mammalian and yeast Lgl (hLgl and Sro7) have been shown to interact with t-SNAREs, (Syntaxin in mammals and Sec9 in yeast) and the exocyst complex (Gangar et al., 2005; Zhang et al., 2005). During abscission, the SNARE complex is necessary for the fusion of vesicles that are targeted to the furrow by the exocyst complex (Eggert et al., 2006). If this interaction is conserved in
Drosophila, than Lgl may be the link between targeted membrane vesicles and the cleavage furrow during abscission.

Anillin is indirectly implicated in vesicle trafficking to the ingression furrow as it localizes septin to the contractile ring (Field et al., 2005). Septin is necessary for vesicle trafficking to the membrane (Beites et al., 1999; Eggert et al., 2006), and it might help shape the plasma membrane at the intercellular bridge, and regulate vesicle fusion during abscission. It is likely that Septin is performing the same task in columnar epithelia through its association with the contractile ring.

**Abscission**

The presence of circular structures in late telophase outlined by Zipper-GFP, independent of Anillin was intriguing, and was reminiscent of the intercellular bridge marker, Cindr, in the imaginal wing disc (Haglund et al., 2010). The ring formed by Zipper also supports an excision model for abscission, where the two opposing sides of the ingression furrow flanked by Zipper are negotiated into a circularized membrane, while the ends of the new bicellular membrane is annealed. Determining whether components of the exocyst, SNARE, or Bruce complexes (Gromley et al., 2005) are associated with the Zipper-GFP ring will help determine if this rig is a result of abscission. As this stage was not assayable in NrxIV or Gli RNAi expressing cells, it is unknown whether its localization or formation is affected. One expectation is that this ring would be absent in NrxIV RNAi expressing discs, as cells do not reach this stage.

The establishment of two new TCJs at the late ingression furrow suggests that Gli has a role. Another avenue for examining abscission and the Zipper-GFP ring is determining whether it is affected in Gli RNAi expressing cells. If Gli RNAi cells are unable to complete abscission, then
there could be an accumulation of the Zipper-GFP ring, or late telophase C cells, after Anillin had already dissociated from the furrow. Assaying for this structures formation, mislocalization and distribution in Gli RNAi expressing cells would help identify whether Gli at the new TCJ is necessary for the completion of cytokinesis, and whether this ring represents a new structure associated with abscission.

Vertebrate tight junctions

If the SJs and Gli are necessary for cytokinesis and maintain the plane of cell division in Drosophila, then a logical question is whether the TJs and Tricellulin perform similar roles in vertebrate epithelia. Evidence already suggests that the TJs are involved in cell division in architecturally complex vertebrate epithelia (such as MCDK cells), as cell division occurs as the TJs and the claudins are associated with midbody (Reinsch and Karsenti, 1994; Kojima et al., 2001). This supports the idea that the localization of cell division to apical permeability barriers may represent a conserved mechanism. Since Tricellulin is analogous to Gli in its role at the tTJ, Tricellulin, it may also be involved in maintaining the plane of cell division. If Tricellulin does not maintain the plane of cell division, then this would indicate that this function is specific to Gli at the TCJ and not a structural phenomenon of the TCJ of the permeability barrier in general, or that another component at the tTJ or TJ performs this function in vertebrates.

Regardless of whether the vertebrate tight junctions are necessary for cytokinesis, further study of cell division in Drosophila columnar epithelia and other architecturally complex cells will provide insight into the general organization of cell division, not easily discernable in more compact cells.
**Figure 5.1 Cytokinesis in architecturally complex columnar epithelia.**

A. Cartoon illustrates the progression from ingression furrow to SJ tunnel. Telophase A-C cells are shown at the apical and basal side of the SJs. midbody (dark blue), nuclei (charcoal), Gliotactin (green), septate junctions (red lines), contractile ring (bright red). In telophase A-B the ingression furrow is indicated with light blue. In telophase C light blue indicates the new bicellular membrane (apical) and the septate junction tunnel (basal).

Telophase A: The ingression furrow forms visible as two sites of contraction through the SJ domain. Anillin localizes to this region as well. The nuclei are still localized to the SJ domain. Midbody markers have started to accumulate between the separated nuclei.

Telophase B: The ingression furrow is more structured and the contractile ring consists of two rings located at either end of the furrow. The midbody is condensed and the nuclei have begun to descend.

Telophase C: A new bicellular SJ has formed apical to the midbody and the contractile ring and the ingression furrow have contracted to a smaller diameter around the midbody. This marks the transition from the ingression furrow to the SJ tunnel. Gliotactin is associated with the basal region of the SJ tunnel and either end of the new membrane.

B. Cartoon illustrates a permissive model where lethal giant larvae (Lgl) acts as the mediator between the septate junctions and the contractile ring. Lgl (yellow) at the septate junctions binds Zipper (red) at the ingression furrow linking the membrane to the contractile ring.

C. Cartoon illustrates an inhibitory model where lethal giant larvae (Lgl) is downregulated at the ingression furrow to allow for activated Zipper. Lgl (yellow) at the septate junctions binds Zipper (red) at the ingression furrow linking the membrane to the contractile ring.
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